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13. ABSTRACT ***(Maximum 200 Words)***
Transforming growth factor-beta (TGF-β) is a potent growth inhibitory polypeptide hormone. Although the loss of this negative proliferative signal is often seen in the deregulated growth of early cancer formation, the TGF-β mediated intracellular pathways that control cell growth remain largely unknown. We propose three aims to 1) elucidate the functional role of Smads in TGF- β signaling, 2) assess their contribution in regulating cell proliferation and 3) to provide evidence that Smad3 acts *in vivo* as a tumor suppressor of early breast cancer formation. In the first year of funding, we have provided evidence that the Smads are specific sequence DNA-binding proteins, and functionally interact with other transcription factors and the p300/CBP coactivator family of proteins to coordinate disparate pathways in the regulation of specific genes. Towards the goal of the second aim, we demonstrated that Smad3 is an essential component of TGF-β mediated growth inhibition in fibroblasts and epithelial cells, and that cyclin D1 and c-Myc are Smad3 dependent TGF-β repressible target genes. The last aim to evidence that Smad3 may be an *in vivo* tumor suppressor will be completed with the use of the Smad3 null mouse model, carcinogen treatment and crossing into the APCmin mouse background.

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FOREWORD

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Joshua P. Frederick 7/30/00

PI - Signature Date

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Introduction

Transforming growth factor-beta (TGF-β) clearly plays a complex role in the physiology of mammary gland development and autoctrine/paracrine homeostasis, and the pathophysiology of breast carcinogenesis. These global effects of TGF-β activity are thought to largely stem from the ability of this hormone to modulate the transcriptional activity of a variety of target genes that are classicaly divided into two subsets: those involved in cell cycle regulation and those involved in extracellular matrix modulation. The ability of TGF-β to potently suppress the proliferation of normal breast epithelial cells may be central to its putative role in tumor suppression of early stage breast cancers. However, it is important to note that in later stage breast cancers, in which epithelial cells are often refractory to the antiproliferative effects of TGF-β, the modulation of the extracellular matrix (ECM) by the TGF-β signal may contribute to breast cancer progression. Thus, the elucidation of the mechanisms by which TGF-β is able to exert these effects are intensely researched. The Smad family of proteins are the most well characterized mediators of the TGF-β signal. Upon phosphorylation by the TGF-β receptor complex, Smads 2 and 3 heterodimerize with the central partner of Smad signaling, Smad4, and shuttle to the nucleus. (Figure 1) This induced nuclear translocation suggested a potential role for the Smad family of proteins in transcriptional regulation; a role that has been largely established from much research in the last three years. The goal of the original proposal was to further define the role of the Smads as transcriptional regulators with particular attention to target genes involved in cellular growth inhibition, and thus, genes potentially involved in the tumor suppression of early breast cancers. The successful completion of these studies will provide further understanding of Smad mediated signaling with an emphasis on the role of these effector molecules in growth inhibition and breast tumor suppression, and thus potentially provide a theoretical framework from which new breast cancer therapeutics may be devised.

Body

The first technical objective of the original proposal, to **determine the mechanism through which the Plasminigen Activator Inhibitor-1 (PAI-1) gene is regulated by Smad3/4 and/or Smad2/4**, was chosen because PAI-1 was a well established TGF-β target gene that was shown to be synergistically activated by overexpressed Smad 3 and 4 in a transcriptional reporter assay (23). In addition, we recently demonstrated that a Smad3/4 complex could physically interact with specific DNA response elements in the PAI-1 promoter, and that Smad4 alone is able to directly a specific sequence of DNA, CAGACA (22). Thus, although PAI-1 is a major constituent of the ECM, and not necessarily a mediator of the TGF-β antiproliferative signal, it was chosen as a model gene to study the transcriptional activity of the Smads. Although progress was made in the completion of the first technical objective, various other groups were able to publish their findings of this line of investigation first. Within the last year, four reports were published that defined Smad3/4 binding sites within the PAI-1 promoter, and characterized these sites as functionally essential in the TGF-β mediated transactivation of PAI-1 promoter reporter constructs (5,8,18,20). We and others have shown that Smad 2 and 4 overexpression can also transactivate PAI-1 reporter constructs, however the mechanism by which this is accomplished seems to be distinct from that mediated by Smad3 and 4 and is currently unknown. We have been able to define a distinct region of the PAI-1 promoter through which Smad 2 and 4 are able to transactivate this gene, but have not been able to demonstrate direct binding of Smad2/4 to any promoter sequence (unpublished data). The following factors have lead to an emphasis on Smad 3 signaling in this proposal, as opposed to Smad 3 *and* 2 signaling: the progress made in defining Smad3/4 complexes in the mediation of TGF-β transcriptional control, and the availability of Smad 3 null cells in our laboratory. Although random degenerative oligos were obtained to complete part C of the first technical objective, another group first published the completion of this study (23).

From these findings and those obtained from the characterization of other TGF-β responsive genes, the "CAGA box" was established as a Smad3/4 binding element. (5,8,17,18,20,21,22,23). One such study in which I contributed, was the demonstration that Smad3 and 4 and the transcription factor family of proteins, AP-1, synergize in the transcriptional activation of the cJun promoter by TGF-β (21, attached ref. 1). This study further developed the role of Smad 3/4 as a transcriptional regulator with the ability to coordinate its activity with that of other transcriptional factors, and provides a conceptual basis for how the TGF-β signal can be integrated with other disparate signals on the same gene. Furthermore, in collaboration with other members of our laboratory, we have

demonstrated that this transcriptional synergy between Smads and AP-1 is likely through the direct physical interaction of Smads and AP-1 family members (10, attached ref. 2). Finally, we have shown that the TGF-β induced phosphorylation of Smad3 facilitates its interaction with the transcriptional coactivators p300/Creb Binding Protein (CBP) (16, attached ref. 3), which provides molecular justification for the choice of one of the animal models that will be proposed in the new technical objective 3. Thus, although the exact aims delineated in the original technical objective one were largely completed by other groups, my efforts, and thus your funding, in these other related studies has contributed to a better understanding of how Smads transduce the extracellular TGF-β signal into transcriptional regulation of target genes.

The identification of Smad target genes involved in TGF-β mediation growth regulation is the focus of the second aim, which is now a combination of the original technical objectives 2 and 3 with the following outlined modifications. We have recently created a Smad3 null mouse from which we have generated Smad3 null fibroblast and epithelial cells. With these tools we have definitively shown that Smad3 plays an integral role in TGF-β mediated growth arrest in fibroblasts and cells of epithelial and lymphoid origins (4, attached ref. 4 and unpublished data). Thus, although Smad2 and 4 have been characterized as *bonafide* tumor suppressors due to their characterized mutations in human cancers, it is conceivable that Smad3 could play a tumor suppressor role in its antiproliferative capacity. Technical objective 2 will be carried out as described with the exception that primary fibroblasts and epithelial cells derived from our wildtype Smad3 animals will be used in the completion of these studies due to the fact that they should not harbor undefined mutations commonly generated in cell lines. We have already established adenovirus constructs expressing Smads 2, 3 and 4, and have successfully infected primary epithelial cells. Using the aforementioned primary cell systems, as well as the Smad expressing adenovirus constructs, we have made the following steps in the completion of technical objective 2.

Since the last annual report, it has been discovered that one mechanism by which TGF-β inhibits the growth of primary fibroblasts is through the downregulation of the G1-phase type cyclin, cyclin D1 (Figure 2B). 24 hours of TGF-β treatment results in the reduction of cyclin D1 protein levels, and it was demonstrated that this induced reduction of cyclin D1 is Smad3 dependent. Furthermore, the basal levels of cyclin D1 in Smad3 null cells is markedly increased compared to wildtype fibroblasts, which is possibly due to endogenous TGF-β loss of function. Other previously identified, cell cycle related targets of TGF-β signaling (eg. p15, p21, cdc25a) were unaltered in wildtype cells, and thus were of no interest to pursue in this cell system (Figure 2A). Additionally, primary epithelial cells were isolated from neonatal epidermis, as epithelial cells are a more appropriate cell type in which to study the TGF-β antiproliferative signal. Wildtype

epithelial cells are growth inhibited upon TGF-β treatment 86% compared to only 35% in Smad3 null cells (Figure 3D). In these cells the c-Myc oncoprotein is repressed at both mRNA and protein levels by a Smad3-dependent mechanism (Figure 3A,B,C). Furthermore, adenovirus mediated overexpression of Smad3 and Smad3/4 are sufficient for repression of c-Myc mRNA (Figure 4).

Thus, we have demonstrated in primary cells that Smad3 is an integral player in the TGF-β antiproliferative signal, and that the TGF-β mediated repression of cyclin D1 and c-Myc are Smad3 dependent. We are currently attempting to define the exact mechanism(s) by which Smad3 is able to repress these target genes, and thus whether this observed repression is through a direct effect mediated by Smad3 or through undefined Smad3 dependent secondary effects. The ability of Smad3 to transduce the TGF-β antiproliferative signal, possibly through the repression of cyclin D1 and c-Myc, may be involved in TGF-β mediated tumor suppression of early breast cancers.

The original technical objective 3, to **identify new target genes regulated by the Smads**, will be carried out as part of the modified technical objective 2. However, this objective will be accomplished with a different technical methodology that that originally proposed, i.e. the SAGE (Sequential Analysis of Gene Expression) system. Due to the availability of Smad3 null cells, these cells will be used to identify new target genes regulated by Smad3. Wildtype and Smad3 null cells will be incubated in the presence and absence of TGF-β, and mRNA isolated after 1-2 hours of treatment. The isolated mRNA will then be used to hybridize with commercially available cDNA microarray blots, eg. the microarrays that Genome Systems has developed, which includes over 18,000 cDNAs. This system will be employed in place of the SAGE system because similar protocols have been previously used in our laboratory and are less technically challenging. Results will be analyzed with and compared by the company's corresponding computer software. Smad3 target genes will be readily identified by comparing results obtained from TGF-β treated wildtype cells to those from Smad3 null cells. The comparison between untreated wildtype and Smad3 null cells will also be informative. The completion of this subaim of technical objective 2 is pending allocation of additional funding to purchase the Genome Systems microarray technology.

The new third technical objective, which was initiated in the second year of funding and will be more intensely pursued in the final third year, will be **to define the *in vivo* role of Smad3 in mouse models of breast cancer**. The availability of the Smad3 null animals in our laboratory provides an extremely valuable tool to address this line of investigation, which I am now taking advantage of in the completion of this proposal. First the mammary tissue of variously aged, female Smad3 null mice will be compared to that of corresponding wildtype littermates. The tissue will be examined histologically for signs of ductal epithelium hyperplasia, as well as signs of more advanced carcinomas.

Although it is possible that no signs of a predisposition for breast cancer will be observed in Smad3 null animals, previous work with TGF-β receptor transgenic animals suggests that the Smad3 null animals may indeed present with ductal epithelial hyperplasia and/or carcinoma. It was demonstrated that the overexpression of a TGF-β type II dominant negative receptor in the mammary epithelium of transgenic mice, which inhibits endogenous TGF-β signaling, results in epithelial hyperplasia (7). In a separate study of similar, independently derived transgenic mice, it was shown that carcinogen, 7,12-dimethylbenz-[*a*]-anthracene (DMBA), induced mammary tumorigenesis was enhanced compared to wildtype controls (3). Furhtermore, transgenic mice harboring forced overexpression of the ligand TGF-β1 in mammary epithelium, which results in activation of the TGF-β pathway, are resistant to DMBA induced breast cancer formation. Given the possibility that Smad3 null mice alone may not present with mammary epithelium hyperplasia or evidence of breast cancer formation, the Smad3 null mice will be treated with DMBA and analyzed in a similar fashion as that of untreated mice. Although, the DMBA studies may not be completed in the final year of funding, I will initiate this line of inquiry to be completed by other members of our laboratory.

In preliminary histological analysis of Smad3 null mammary ductal epithelial, there has been no overt evidence of hyperplasia, although we are currently assessing *in vivo* rates of breast epithelial proliferation via BrdU incorporation. In addition, we will also determine if cyclin D1 and c-Myc protein levels are elevated in Smad3 null breast epithelium through immunohistochemistry analysis given that these genes are repressed in a Smad3 dependent fashion in cultured primary cells. We have already determined that cyclin D1 and c-Myc protein levels are markedly upregulated in Smad3 null colonic epithelium, indicating these two genes may be important target genes repressed by Smad3 action *in vivo*.

Finally, the Smad3 null animals will be crossed into a genetically altered mouse line predisposed to breast cancer development in further efforts to uncover a potential role of Smad3 in breast cancer tumor suppression. These experiments will be conceptually similar to those proposed with DMBA induced breast cancer formation, yet are more controlled as the introduced mutations will be engineered, whereas DMBA treatment can potentially result in multiple, unknown mutations. We will focus our efforts on the characterization of animals generated from introducing the Smad3 null background into the APCmin mouse model.

The APCmin mouse harbors an inactivating APC mutation and APC heterozygote mice develop colonic adenomas with high penetrance, and mammary tumorigenesis with lower incidence (12). APC is a tumor suppressor protein that functions in the *wnt* signaling pathway as a negative regulator. It serves to sequester β-catenin in the cytoplasm, and targets this transcriptional activator for degradation. With a positive *wnt*

signal, or inappropriate activation of this pathway in a cancerous cell, β-catenin translocates to the nucleus to from a functional transcriptional complex with the DNA-binding protein tcf-4 (13).

The *wnt* and TGF-β pathways have long been linked epigenetically in both antagonistic and synergistic interaction in developmental studies in *Xenopus* and *drosophila*. We have recent evidence that the *wnt*/APC/β-catenin/tcf-4 pathway and the TGF-β/Smad3 pathway may be antagonistic on the molecular level, and thus possibly on the more global level of cancer development. Smad3 was found to interact with a tcf family member in a yeast two-hybrid screen and directly interact with *in vitro* translated tcf-4 (data not shown). The relevance of this interaction is supported by the recent demonstration that Smad4, β-catenin and Lef1/tcf directly interact to synergistically activate a target gene involved in a developmental context (25). In the context of cancer, it was demonstrated that the TGF-β and wnt pathways may act antagonistically as compound heterozygosity of APCmin and Smad4 results in enhance malignancy of colorectal carcinoma (27). It was recently shown that cyclin D1 and c-Myc are direct target genes of β-catenin/tcf-4 transcriptional activation (26, 28, 29, 30). These studies in conjunction with our findings that these genes are repressed by TGF-β in a Smad3 dependent manner raises the possibility that these pathways may directly antagonize the function of one another in the transcriptional regulation of c-Myc and cyclin D1 (Figure 5). Although the inappropriate activation of this pathway is classically associated with colon cancer development and tcf-4 was first shown to be highly expressed only in colonic epithelium, a recent report demonstrates comparable levels of expression in mammary epithelium (1). Furthermore, the inappropriate activation of this *wnt* pathway has been linked to poor prognosis in certain breast cancers (26). It is thus hypothesized that this proposed crossing of mice, which would harbor the oncogenic activation of the *wnt* pathway and the loss of the potentially tumor suppressive Smad3 pathway, will result in more severe breast and colon cancer formation than that seen in APC heterozygote animals alone. The molecular mechanisms involved in the potential antagonistic relationship between the TGF-β and wnt pathways will be studied on a cellular and molecular level with the aid of cells isolated from the aforementioned mouse crossing,and retroviral constructs overexpressing β-catenin, tcf-4 and Smad proteins. These retrovirus constructs are currently under construction.

Figure 1. Introduction.

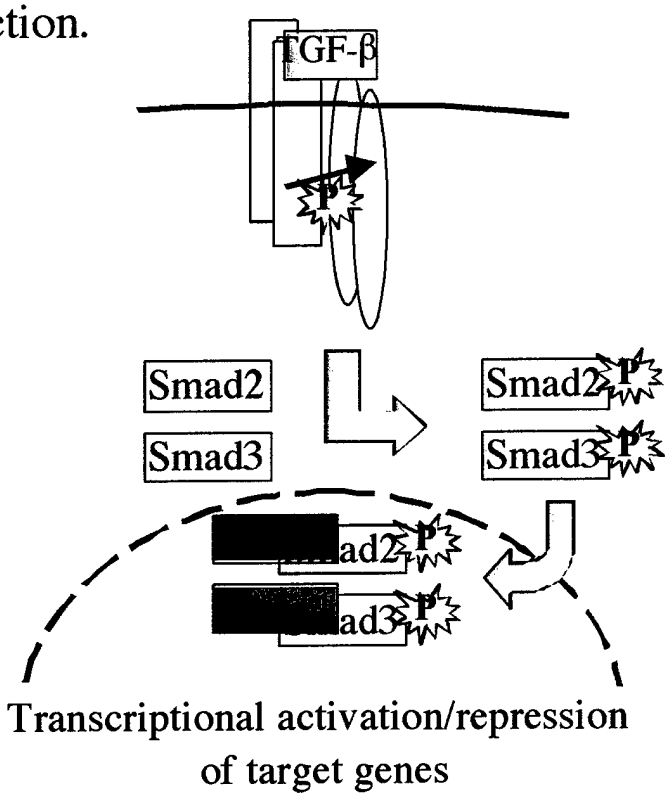


Figure 2A.

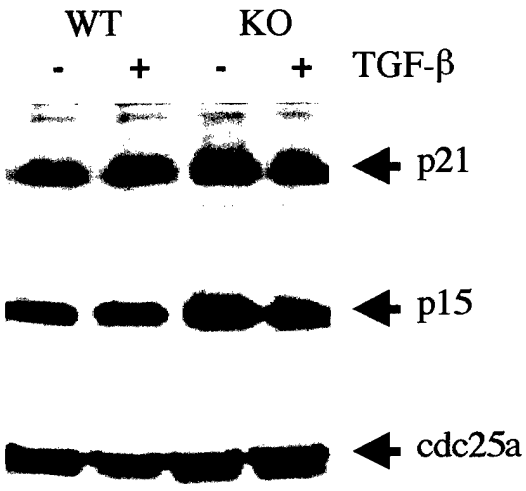


Figure 2B. TGF-b mediated repression cyclin D1 is Smad3 dependent in MEFs.

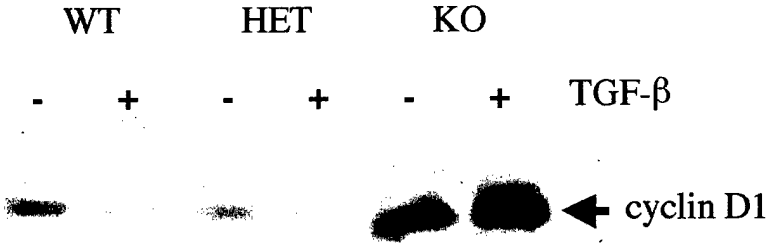


Figure 3. TGF-β mediated growth inhibition and c-Myc repression is Smad3 dependent in keratinocytes.

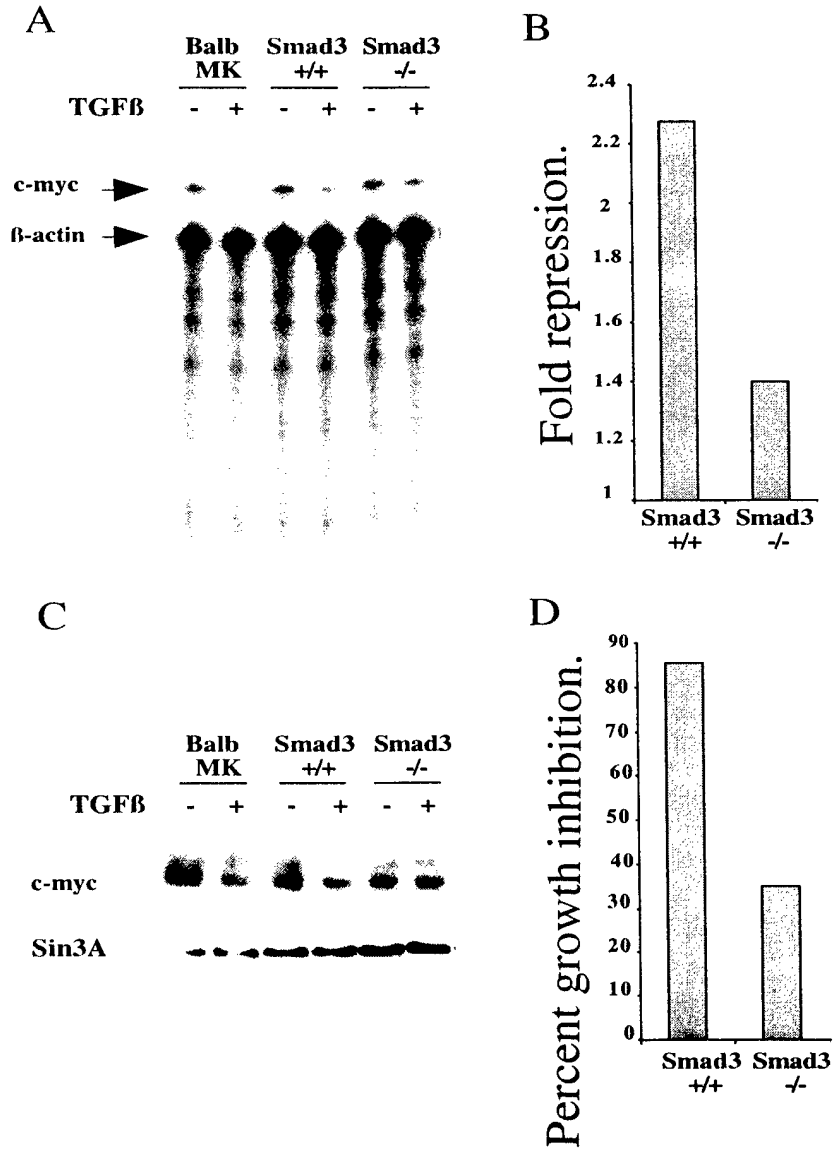
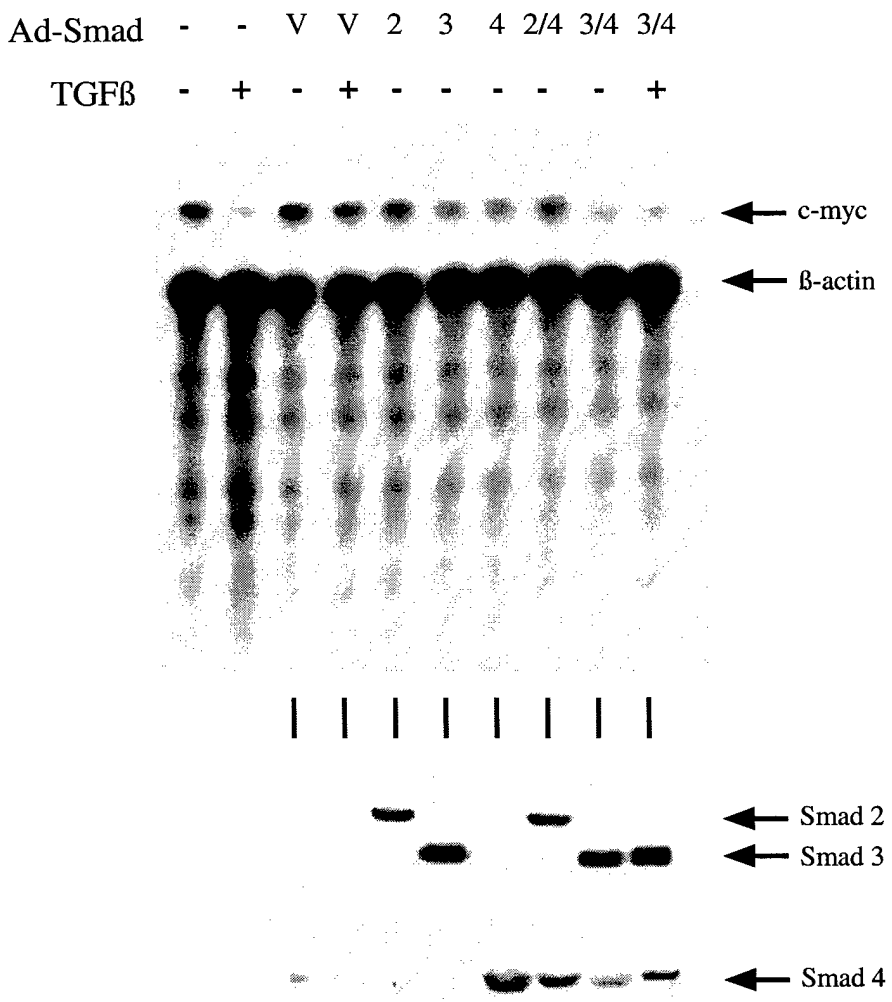


Figure 4. Adenoviral transfer of Smads repress c-Myc mRNA levels.

A



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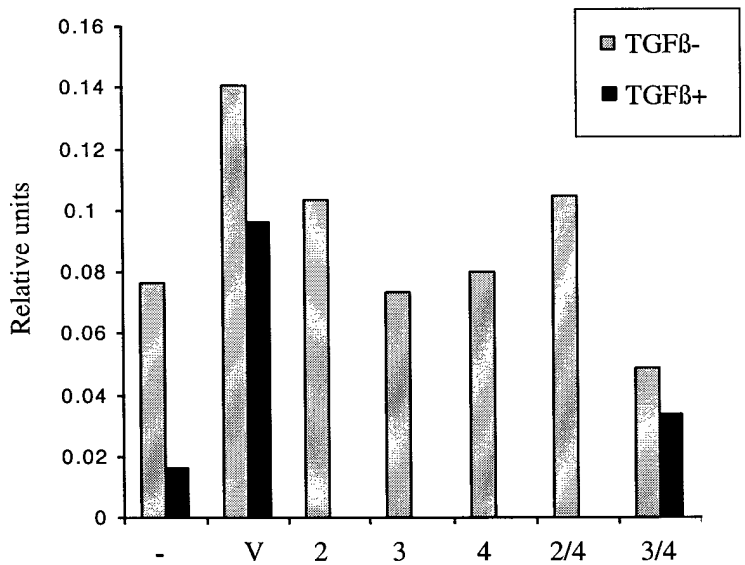

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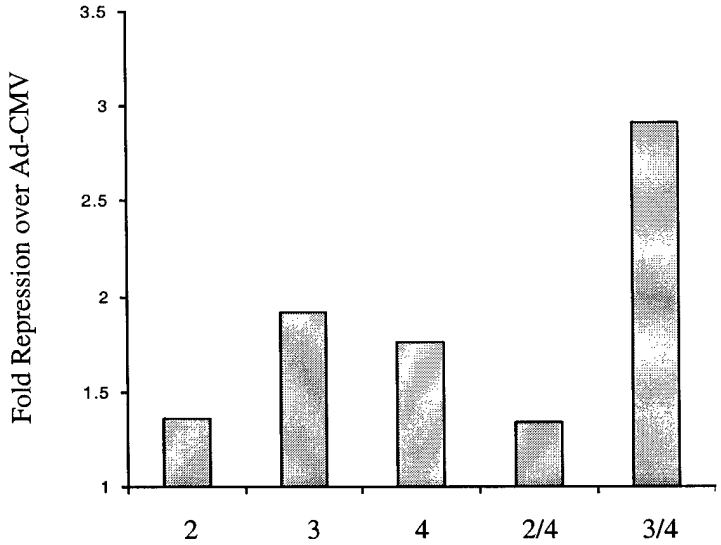
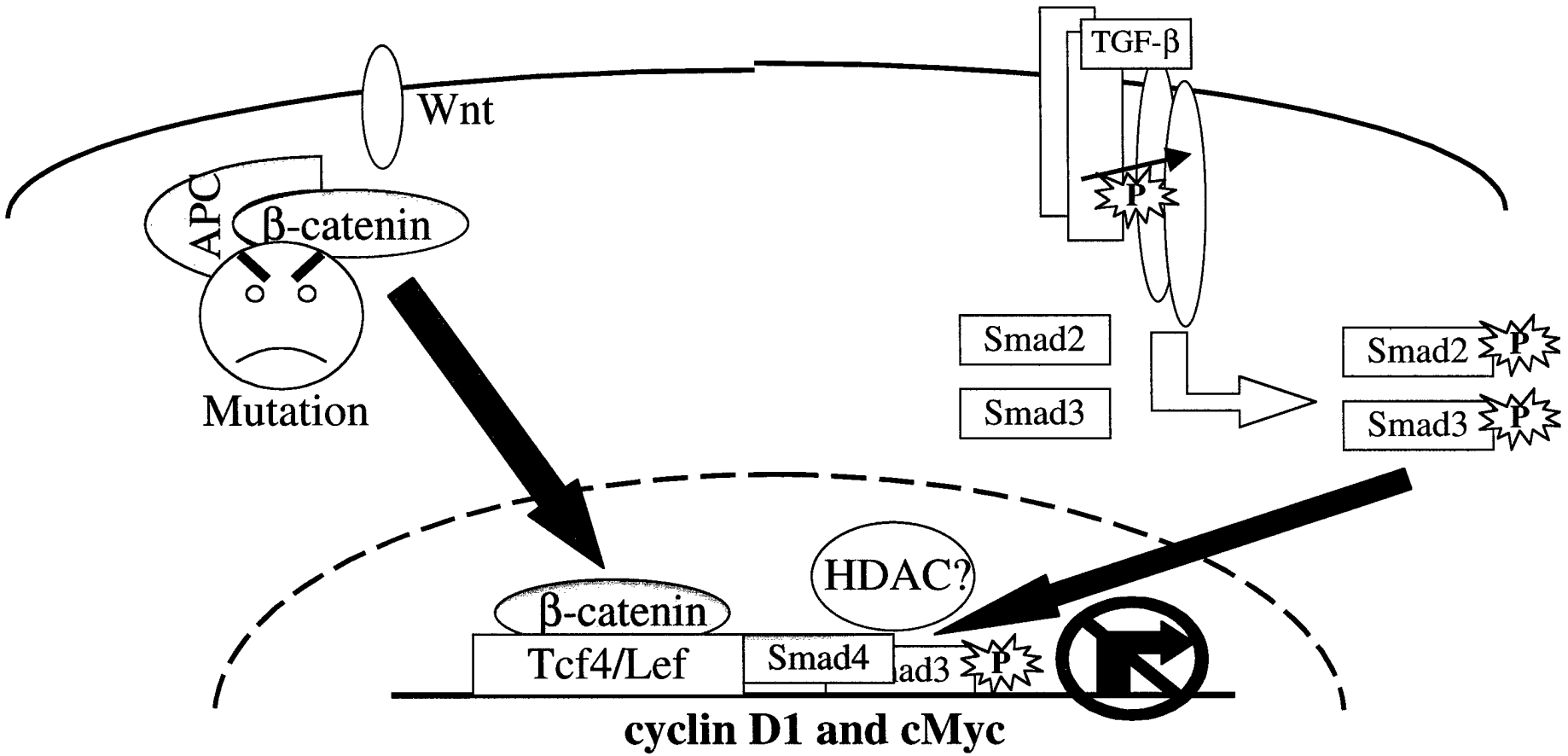


Figure 5. A model for antagonistic crosstalk between the TGF-β and wnt signaling pathways.



Key Research Accomplishments

- Smad3 and 4 and the transcription factor family of proteins, AP-1, synergize in the transcriptional activation of the cJun promoter by TGF-β.

- Smads and the transcription factor family of proteins, AP-1, directly interact with one another.

- TGF-β induced phosphorylation of Smad3 facilitates its interaction with the transcriptional coactivators p300/Creb Binding Protein (CBP).

- Smad3 is an integral component of TGF-β mediated growth inhibition in primary epithelial cells and fibroblasts.

- Smad3 is essential for the TGF-β mediated repression of cyclin D1 and c-Myc in primary cells.

Reportable outcomes

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   * Denotes equal contribution

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Smad3-Smad4 and AP-1 Complexes Synergize in Transcriptional Activation of the c-Jun Promoter by Transforming Growth Factor β

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Transcriptional regulation by transforming growth factor β (TGF-β) is a complex process which is likely to involve cross talk between different DNA responsive elements and transcription factors to achieve maximal promoter activation and specificity. Here, we describe a concurrent requirement for two discrete responsive elements in the regulation of the c-Jun promoter, one a binding site for a Smad3-Smad4 complex and the other an AP-1 binding site. The two elements are located 120 bp apart in the proximal c-Jun promoter, and each was able to independently bind its corresponding transcription factor complex. The effects of independently mutating each of these elements were nonadditive; disruption of either sequence resulted in complete or severe reductions in TGF-β responsiveness. This simultaneous requirement for two distinct and independent DNA binding elements suggests that Smad and AP-1 complexes function synergistically to mediate TGF-β-induced transcriptional activation of the c-Jun promoter.

Transforming growth factor β (TGF-β) is a multifunctional cytokine with a wide range of physiological as well as pathological effects (reviewed in references 31 and 42). Its physiological roles include inhibition of the proliferation of a variety of cell types, negative regulation of the immune system, and positive regulation of extracellular matrix deposition. Dysregulation of these processes can result in various fibrotic as well as malignant diseases. Indeed, many late stage cancers have lost expression of TGF-β receptors, which renders them resistant to TGF-β-mediated growth inhibition (19, 29, 36, 38, 50, 55); restoration of TGF-β pathways in these cells can often restore growth inhibition and decrease the malignant phenotype. TGF-β-mediated immune system suppression and stimulation of extracellular matrix (ECM) production may also contribute to tumor-promoting effects.

Regulation of transcription of specific sets of genes by TGF-β mediates many of these physiological roles. Upregulation of two cyclin-dependent kinase inhibitor genes, p21 and p15, has been shown to mediate TGF-β-induced growth arrest in certain cell types (7, 12, 41), while upregulation of ECM genes, including plasminogen activator inhibitor 1 (PAI-1), fibronectin, and collagen genes, may mediate other effects of TGF-β. However, many of the genes regulated by TGF-β are also regulated by a variety of other signals, including some signals which appear to play very distinct roles at the physiological level. Of particular note is a subset of TGF-β immediate-response target promoters, including the TGF-β1 ligand gene and most of the TGF-β-responsive extracellular matrix genes, in which AP-1 binding sites have been found to be involved in mediating the TGF-β signal (4, 22, 51). The use of AP-1 sites in TGF-β-dependent transcription has been particularly puzzling, given the extensively described mitogenic signaling pathways which also activate transcription through AP-1; the mechanism by which TGF-β regulates these promoter sequences has not been clarified. An additional level of complexity is introduced by the regulation by TGF-β of the expression of AP-1 family members themselves. This suggests that there can be both primary and secondary effects on transcription through AP-1 by TGF-β.

The regulation of AP-1 transcription factors by TGF-β varies with the specific family member and with cell type. The upregulation of c-Jun transcript occurs in a wide range of cell lines derived from both normal and transformed cells. This response to TGF-β is early and immediate, with mRNA induced within 15 to 30 min. While cycloheximide studies have been inconclusive, due to the inducing effects of the cycloheximide itself on c-*jun* transcription, the time course of induction strongly suggests that this gene could be a primary target of TGF-β (24, 26, 39), which is supported by the current study describing specific promoter elements capable of mediating TGF-β's induction of c-Jun.

The model for TGF-β activation of transcription continues to undergo rapid development. The Smads are a recently identified family of proteins which operate downstream of various members of the TGF-β superfamily (reviewed in references 13, 14, 23, 30, and 37). Smad2 and Smad3 are downstream effectors of the TGF-β signaling pathway. Upon ligand binding, they are phosphorylated by the TGF-β type I receptor kinase and translocate to the nucleus in a complex with Smad4 (28, 35, 59). Recent work has identified a potential consensus Smad3-Smad4 DNA binding site, GTCTAGAC (58), by random oligonucleotide screening, as well as similar sequences in the PAI-1 promoter (9), the engineered TGF-β-responsive reporter construct, p3TP-lux promoter (57), the JunB promoter (18), and the COL7A1 collagen promoter (54). It was found that four copies of the oligonucleotide consensus site or nine copies of the PAI-1 site could confer TGF-β responsiveness on a minimal promoter. In addition, mutation of all three putative Smad3-Smad4 binding sites in the PAI-1 promoter could eliminate TGF-β responsiveness of that promoter in HepG2 cells.

Although these studies demonstrate the importance of

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Smad3-Smad4 binding sites in the mediation of TGF-β responsiveness, they do not fully address the issue of whether binding elements for other transcription factors are also required for TGF-β-mediated transcriptional activation of target promoters. Biochemical and overexpression studies have demonstrated that Smads are capable of functional interaction with Sp1 (33) and with AP-1; in fact, direct physical interaction between Smads and AP-1 family members has been demonstrated in model systems (27, 60). Cooperation between Smad2-Smad4 complexes and FAST-1 has been demonstrated at an activin responsive *Xenopus* promoter (2, 3). Finally, a very recent study reports that a binding site for the transcription factor muE3 (TFE3), as well as one for Smad3 and Smad4, is required for TGF-β-mediated transcription of a reporter controlled by a specific region of the PAI-1 promoter (16).

While the TGF-β-responsive elements in the c-Jun promoter have not previously been characterized, extensive work has established the importance of two AP-1/CRE sequences in the c-Jun promoter in regulation by phorbol-12-myristate-13-acetate (TPA), serum, UV, E1A, and interleukin 1 (IL-1) (1, 15, 34, 43, 53). Furthermore, a reporter construct controlled by the −79 to +170 sequence of the c-Jun promoter, which contains only the more proximal AP-1/CRE site (−71 to −64), has proved sufficient for a maximal response to most of these signals. Interestingly, none of these stimuli appears to change the occupancy of any identified binding sites in the c-Jun promoter. Thus, the prevailing model of activation by these other signals is thought to be through modification of a constitutively promoter-bound complex, in most cases c-Jun-ATF-2.

Here, we identify two DNA binding elements within this −79 to +170 region which are indispensable in TGF-β-mediated induction of c-Jun: the proximal AP-1/CRE site known to be important for the response to several other signals, and a novel Smad3/Smad4 binding site. Mutation of either site alone is found to abolish or severely reduce promoter upregulation by TGF-β, despite the presence of the remaining element. Our results suggest that the two complexes can cooperate synergistically in activating TGF-β-mediated transcription of this c-Jun promoter region.

MATERIALS AND METHODS

Antibodies and reagents. Human TGF-β1 was from R&D Systems. Rabbit polyclonal antisera recognizing Smad3 and Smad4 were generated in this lab. Smad3 antiserum was raised against a specific Smad3 peptide (DAGSPNLSPNPMSPAHNNLD), while Smad4 antiserum was raised against full-length human glutathione *S*-transferase–Smad4.

Cell culture. Mink lung epithelial cells and primary mouse embryo fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, penicillin, and streptomycin. Immortalized human keratinocyte cells (HaCaT) were grown in MEM supplemented with 10% FBS, penicillin and streptomycin, and 20 mM L-glutamine. Primary fibroblasts were harvested from day-14 embryos. Embryos were mechanically disrupted by passage through an 18-gauge needle and plated on gelatin-coated 10-cm-diameter plates in DMEM with 20% heat-inactivated FBS, penicillin, streptomycin, and gentamicin (Gibco BRL, Gaithersburg, Md.). When confluent, cells were trypsinized and further maintained in DMEM with 10% FBS. The targeted disruption of the Smad3 allele in these mice and the characterization of their phenotype are described elsewhere (6).

Plasmid constructs. Flag-tagged human Smad4 was a generous gift from Rik Derynck. Human pCGN Smad3 was described previously (57). The c-Jun luciferase reporter containing the −79 to +170 sequence of the human c-Jun promoter was generously provided by Bin Su (48). The rest of the promoter mutants and 3′ deletion constructs were made by PCR mutagenesis using the following primer sets: as 5′ primers, wild type, 5′CCC AAG CTT GGC CTT GGG GTG ACA TCA TGG GC3′; AP-1/CRE mutant, 5′CCC AAG CTT GGC CTT GGG GAT CCA CCA TGG GCT ATT TTT AGG GG3′; and as 3′ primers, wild type, 5′AAA CTG CAG GCC GAC CTG GCT GGC TGG CTG TGT CTG TCT GTC3′; mutant, 5′AAA CTG CAG GCC GAC CTG GCT GGC TGG CTG TTC CAA GCT CCT TGC CTG ACT CCG3′. A *Hin*dIII site was engineered into the 5′ end of each PCR product, and a *Pst*I site was engineered into the 3′ end of each PCR product. PCR products were subcloned into pGEMT (Promega, Madison, Wis.), and then the *Hin*dIII/*Pst*I fragments were purified on an agarose gel, extracted with a QIAEX II gel extraction kit (Qiagen Inc., Santa Clarita, Calif.), and subcloned back into the *Hin*dIII and *Pst*I sites flanking the 5′ and 3′ ends, respectively, of the −79 to +170 sequence insert in the −79 to +170 luciferase reporter construct. Constructs were verified by restriction digestion with *Hin*dIII/*Pst*I and by sequencing.

Transfection and luciferase assays. Transient transfections were performed with the standard DEAE-dextran method and the luciferase activity was measured 24 h after the addition of 100 pM human TGF-β1 as described previously (8). For all experiments, 3 μg of the indicated luciferase reporter and, when indicated, 1 μg of Smad3 expression vector were used (57). Total DNA was kept constant by using empty pCGN vector. All transfections were normalized to β-galactosidase activity by cotransfection of 0.5 μg of a β-galactosidase (pCMV-β-Gal) expression vector. The luciferase data shown are representative of experiments performed in duplicate in at least three independent experiments.

Nuclear extracts. Nuclear lysates were prepared from control and TGF-β1-treated cells. Briefly, confluent cells from 10-cm-diameter dishes were washed twice with phosphate-buffered saline. After washing, 5 ml of ice-cold hypotonic lysis buffer was added (20 mM HEPES [pH 7.6], 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 25 mM NaF, 25 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and protease inhibitors). The cells were allowed to swell on ice for 5 min before they were scraped and collected. Nuclei were pelleted by centrifugation at 500 rpm in a Beckman swinging-bucket tabletop centrifuge for 5 min and resuspended in 100 to 200 μl of nuclear extraction buffer (hypotonic buffer plus 500 mM NaCl). After incubation and rocking at 4°C, the lysates were cleared of debris by centrifugation.

Western blot analysis. Western blot analysis for c-Jun was performed on nuclear lysates prepared from MEFs. Prior to treatment with TGF-β1 for the indicated times, cells were serum starved for 12 h in DMEM–0.2% FBS. Equal protein amounts were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, and Western blotting was performed with a 1:1,000 dilution of the rabbit polyclonal antibody α-c-Jun (9162) from New England Biolabs, Inc. (Beverly, Mass.).

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed by using 1 to 3 μg of nuclear extracts prepared from untreated cells or cells treated with 100 pM TGF-β1 for 1 h and probes derived from a *Sac*I/*Bam*HI fragment of luciferase construct containing the c-Jun sequence from −79 to +170. The digest produced two fragments of the c-Jun promoter that consist of the sequences from −79 to −19 and −18 to +170. Gel shift conditions were exactly as previously described (57). For supershift analysis of Smads, 2 μl of Smads 3 and 4 immune-phase and preimmune-phase antisera and 2 μg of Smad2 (S-20-X) or Smad4 (C-20-X) antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.), were used. For other supershifts, 2 μg of anti-c-Jun (KM-1-X), ATF-2 (FRBR-1-X and C-19-X), and CREB (C21-X and 24H4B-X) from Santa Cruz Biotechnology, Inc., were used. The sequences of the competitor oligonucleotides used to identify the Smad binding site are shown in Fig. 3A. The sequence of the competitor oligonucleotide containing a CREB/ATF binding site (CRE) was 5′-AGA GAT TGC CTG ACG TCA GGA GCT AG-3′ and its complementary strand. The sequence of the mutated CRE site was 5′-AGA GAT TGC CTG TGG TCA GAG AGC TAG-3′. Where results for only one lysate are shown, similar results were obtained for both HaCaT lysates and mink lung lysates.

RESULTS

TGF-β treatment induces DNA binding of a Smad3- and Smad4-containing complex to a sequence in the 3′ region of the c-Jun promoter. An increase in c-Jun mRNA level has been previously observed within 15 to 30 min of TGF-β treatment in a variety of cell types (24, 39, 49). In order to confirm the induction of endogenous c-Jun by TGF-β, we performed Northern analysis of RNA and Western analysis of nuclear extracts isolated from similarly treated cells. In both mink lung epithelial cells (Mv1Lu) and HaCaT cells, the level of c-Jun transcript increased within 1 h of TGF-β treatment and protein levels were dramatically increased within 2 h of TGF-β treatment (data not shown), confirming that the induction of c-Jun by TGF-β occurs in these cells and is likely to be an early response. The induction by TGF-β was most evident in Mv1Lu cells if the cells were serum starved overnight before addition of TGF-β, since the c-Jun transcript is upregulated by serum.

To aid in defining TGF-β responsive elements in the human c-Jun promoter, we next obtained a luciferase reporter construct under control of the sequence from −79 to +170 of the c-Jun promoter (48). This region, diagrammed in Fig. 1A,

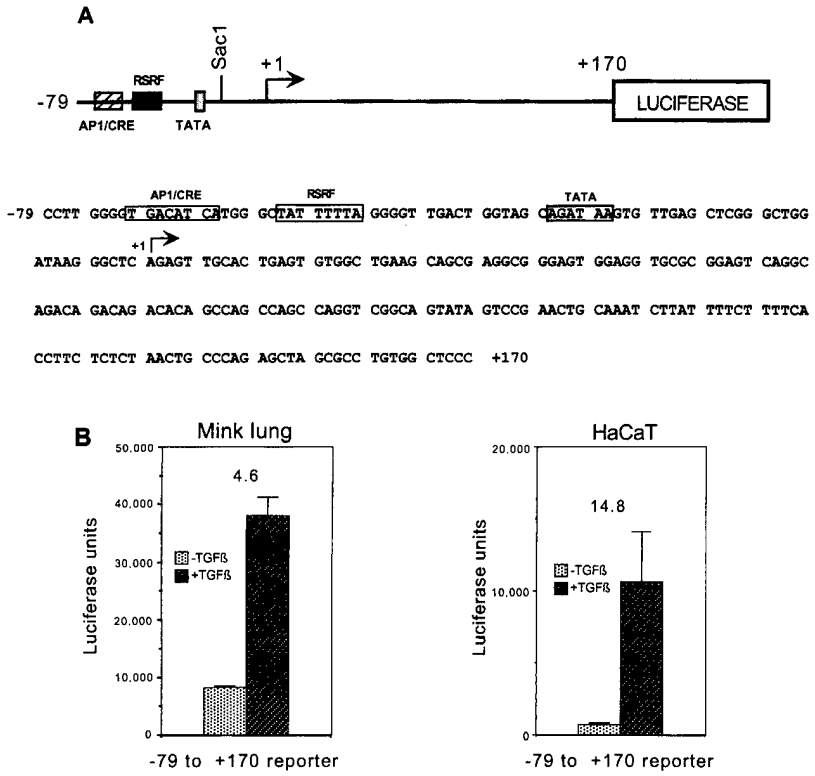

FIG. 1. The −79 to +170 region of the human c-Jun promoter is sufficient to convey TGF-β and Smad3 responsiveness to a luciferase reporter. (A) Schematic representation of the −79 to +170 luciferase reporter. (B) The reporter was transiently transfected into Mv1Lu or HaCaT cells, and TGF-β-induced luciferase activity was measured in relative light units (luciferase units). Fold inductions are indicated above the bars and were calculated by comparing the luciferase activities of cells treated with TGF-β and those of untreated controls.

contains the proximal AP-1/CRE site and the adjacent AT-rich sequence (a putative RSRF [related to serum response factor] site) which is important in epidermal growth factor (EGF) induction of c-Jun, as well as the native TATA box and approximately 170 bp of the sequence 3′ of the start site. As mentioned above, this region was sufficient to convey maximal responsiveness to UV, TPA, EGF, and serum. We transiently transfected this construct into Mv1Lu and HaCaT cells, and measured luciferase activity after TGF-β treatment. As shown in Fig. 1B, the construct was highly responsive to TGF-β, giving 4.6-fold induction in Mv1Lu cells and 14.8-fold induction in HaCaT cells.

Having determined that the −79 to +170 portion of the c-Jun promoter was sufficient to convey TGF-β responsiveness, we next examined whether the mechanism of activation might involve induction of Smad DNA binding to a site in this region. We performed an EMSA by using a 5′ portion or a 3′ portion of the −79 to +170 region as a probe (Fig. 2A) and nuclear extracts from HaCaT cells treated for 1 h with TGF-β. The −79 to −19 probe bound two complexes (small arrows), and no change was observed upon TGF-β treatment (Fig. 2B). On the other hand, the −18 to +170 probe bound a complex that was strongly induced by TGF-β treatment (Fig. 2C). This induced complex appeared within 30 min of TGF-β treatment and was still present at 2 h (data not shown). Using an antiserum specific to Smad3 as well as an antiserum and commercial antibody specific to Smad4, we were able to supershift the induced complex, indicating the presence of both Smad3 and Smad4 in the complex. No supershift was seen with the corresponding preimmune-phase antisera, and a commercially available Smad2 antibody also failed to cause a supershift (Fig. 2C). Similar results were obtained with nuclear extracts from Mv1Lu cells (data not shown).

These results establish the existence of a Smad3-Smad4 binding site contained within the −18 to +170 region of the c-Jun promoter. The binding of Smad3-Smad4 is rapidly induced upon TGF-β treatment, with a time course consistent with that of Smad phosphorylation and subsequent translocation to the nucleus (see references 14 and 23 for reviews). In contrast, the pattern of binding to the −79 to −19 region of the promoter is unchanged upon TGF-β treatment.

The Smad3-Smad4 binding site in the c-Jun promoter is a CAGA triplet located 3′ of the TATA box. In order to identify the Smad3-Smad4 binding site within the −18 to +170 region, four oligonucleotides scanning this sequence (Fig. 3A) were used as cold competitors in the EMSA. Only the +35 to +83 region was found to compete with the binding of the induced complex (Fig. 3B). When the oligonucleotide for this region was cut at a convenient *Hin*fI site and the two halves were compared, binding could be further localized to the +53 to +83 region. Three mutant competitor oligonucleotides of the +53 to +83 region were then designed. We had noted a sequence in the middle of this region, ACAGACAGACAGAC ACAG, which bore great similarity to repeats of the Smad box as identified by previous studies (9, 57, 58) and was recently confirmed by the crystal structure of MH1-Smad3 bound to the CAGA box (47). Therefore, we made mutations to disrupt either this potential Smad binding site or the sequence 5′ or 3′ of it within the +53 to +83 region. Of the three, only the CAGA mutant oligonucleotide had lost its ability to compete

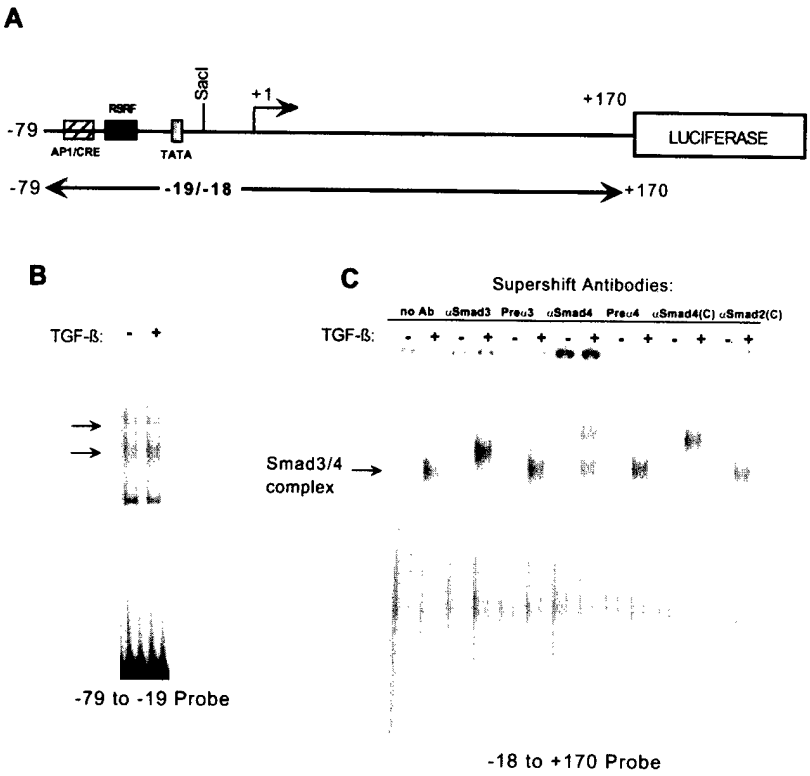

FIG. 2. EMSAs showing induced binding of a Smad3- and Smad4-containing complex to the 3′ region of the human c-Jun promoter. (A) Schematic representation of the −79 to +170 region of the c-Jun promoter showing the probes used for EMSAs. (B) EMSA was performed by using a radiolabeled restriction fragment spanning the −79 to −19 region of the c-Jun promoter and nuclear lysates from either untreated HaCaT cells or HaCaT cells treated with TGF-β1 for 1 h. Two constitutively binding complexes are indicated with arrows. (C) EMSA was performed by using a radiolabeled restriction fragment spanning the −18 to +170 region of the c-Jun promoter and the same HaCaT lysates. A complex that shows binding induced by TGF-β treatment is indicated with an arrow. Supershifts were performed using antiserum against Smad3 or Smad4, shown with their corresponding preimmune-phase antiserum (Preα3 and Preα4) or with commercial antibodies against Smad4 [αSmad4(C)] and Smad2 [αSmad2(C)].

with binding of the induced complex (Fig. 3B and data not shown), indicating that this mutation had disrupted the Smad binding site. Confirming this, a −18 to +170 probe containing mutated CAGA sequence was shown to no longer bind the induced Smad3-Smad4 complex (Fig. 3C). From these experiments we concluded that the Smad3-Smad4 binding site was located at the CAGA repeats within the +62 to +73 region of the c-Jun promoter. These results also established that no other sequences in the −18 to +170 region are absolutely required for DNA binding of the induced complex containing Smad3-Smad4.

Mutation of the Smad3-Smad4 binding site in the c-Jun promoter abrogates responsiveness to TGF-β. Having identified the Smad3-Smad4 binding site in the c-Jun promoter, we set out to determine its importance in mediating the TGF-β response. Using PCR mutagenesis, we created a −79 to +94 wild-type reporter and corresponding −79 to +94 mutant reporters (Fig. 4A). We found that the wild-type −79 to +94 reporter was induced by TGF-β 11-fold and 5.1-fold in HaCaT cells and Mv1Lu cells, respectively. Mutation of the CAGA sequences reduced the response to 2.7-fold and 2.3-fold, respectively (Fig. 4B and C). This demonstrates that the Smad3-Smad4 binding site is important for response to TGF-β in the context of this c-Jun promoter construct.

Although these findings indicate that the identified Smad3-Smad4 binding site is critical in conferring a complete response to TGF-β, there is a small degree of responsiveness which remains after mutation of the Smad3-Smad4 binding site. It is possible that the remaining TGF-β responsiveness is mediated through the AP-1/CRE site at the −71 to −64 region of the c-Jun promoter in a manner similar to that observed in our previous study of the 4XTRE reporter (57).

TGF-β induction and induced complex binding are lost in Smad3-deficient MEFs. The recent creation of Smad3-deficient mice (6) has introduced a powerful new tool for studying the functional importance of Smad3 in isolation. We first compared induction by TGF-β of endogenous c-Jun in primary MEFs established from $Smad3^{+/+}$ and $Smad3^{-/-}$ mice. Primary MEFs were serum starved for 12 h and treated with TGF-β for 4 h, and then nuclear lysates were prepared. As shown by Western blot analysis, induction by TGF-β of total c-Jun protein levels is lost in $Smad3^{-/-}$ MEFs whereas that in $Smad3^{+/+}$ MEFs is intact (Fig. 5A).

We next investigated the ability of exogenous Smad3 expression to rescue c-Jun reporter induction in Smad3 null fibroblasts. $Smad3^{+/-}$ and $Smad3^{-/-}$ MEFs were transfected with −79 to +170 reporter with empty vector or with a Smad3 expression vector. Note that $Smad3^{+/-}$ MEFs express Smad3 and that they activate representative responses to TGF-β to an extent similar to $Smad3^{+/+}$ MEFs (6). The c-Jun promoter was induced approximately threefold by TGF-β treatment in $Smad3^{+/-}$ fibroblasts (Fig. 5B), which is comparable to the fold induction of other TGF-β-responsive promoters examined in these cells (6). However, in $Smad3^{-/-}$ MEFs, TGF-β failed to induce reporter activity (Fig. 5B). Although the uninduced overall activity is lower in the null cells, the full threefold induction by TGF-β was restored upon cotransfection with Smad3. This establishes the absence of Smad3 as the defect

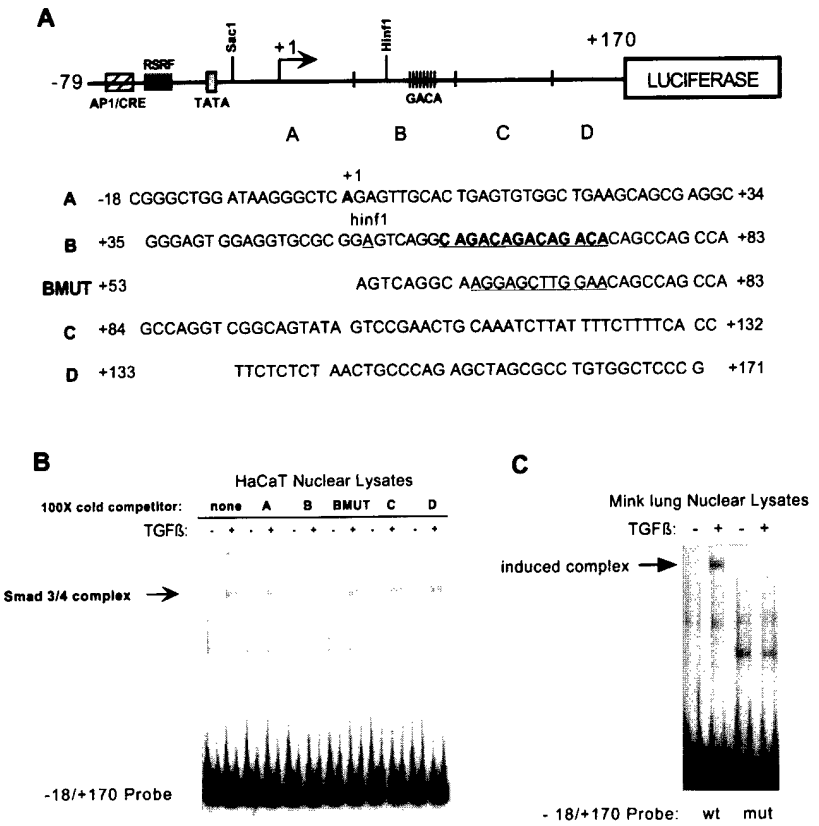

FIG. 3. The Smad3-Smad4 binding site in the human c-Jun promoter is identified as a CAGA triplet located 3′ of the TATA box. (A) Schematic diagram of the −79 to +170 region of the c-Jun promoter. Four oligonucleotide sequences, named A through D, were designed to span the −18 to +170 region of the promoter. An additional oligonucleotide bearing a mutation in a CAGA triplet from +62 to +73 (BMUT) is also diagrammed (see text for additional discussion). The mutation changed the sequence from GACAGACAGACA to AGGAGCTTGCAA. (B) EMSA was performed by using the same −18 to +170 probe and HaCaT lysates as described for Fig. 2. A 100-fold molar excess of unlabeled oligonucleotides was incubated with the nuclear lysates before addition of radiolabeled probe, in order to compete with binding. The induced Smad3-Smad4 binding complex is indicated with an arrow. (C) EMSA was performed by using nuclear lysates from untreated mink lung cells or mink lung cells treated with TGF-β1 for 1 h and the same −18 to +170 probe. Radiolabeled probe was either the wild-type sequence from −18 to +170 or the mutated sequence from +62 to +73 (the CAGA triplet).

responsible for loss of c-Jun promoter activation in these cells, and this result demonstrates that Smad3 is absolutely and specifically required for c-Jun promoter regulation by TGF-β.

Finally, we looked at DNA binding to the Smad3-Smad4 site in the absence of Smad3, to determine whether Smad3 was indeed required for binding of the TGF-β-induced complex. An EMSA was performed by using the wild-type −18 to +170 probe containing the Smad3-Smad4 binding site (see Fig. 2A). Induced complex binding was observed in Smad3$^{+/-}$ fibroblasts, but no induced complex was seen in Smad3$^{-/-}$ fibroblasts (Fig. 5C). This suggests that Smad3 is not only present in but also critical to the formation of the DNA binding complex which is induced upon TGF-β treatment. The correlation between loss of the induced complex and loss of endogenous c-Jun induction and c-Jun reporter activation further supports the importance of the induced Smad3-Smad4 binding complex to TGF-β regulation of c-Jun transcription, as well as firmly establishing the requirement for Smad3 in this process.

Mutation of an AP-1/CRE site can independently abrogate TGF-β responsiveness of the c-Jun promoter. The AP-1/CRE site at −71 to −64 has previously been shown to be important for induction of c-Jun by other signals (1, 15, 34, 53). A consensus AP-1 site was also shown to be not only necessary but also sufficient for TGF-β and Smad responsiveness in the context of a multimerized TRE reporter (57), and mutation of the Smad3-Smad4 binding site in the c-Jun promoter eliminated nearly all but not all TGF-β responsiveness (Fig. 4). While a recent study by Dennler et al. (9) established the importance of three Smad3-Smad4 binding sites in TGF-β regulation of the PAI-1 promoter, it did not address whether the AP-1-like sites present in the promoter (21) may also be important for TGF-β regulation in that context. In order to investigate the importance of the AP-1/CRE site in induction by TGF-β of this c-Jun promoter region, we used PCR mutagenesis to mutate this site in the −79 to +170 reporter. Mutating the AP-1/CRE site abrogated all transcriptional induction of the reporter by TGF-β (Fig. 6A), despite the fact that the Smad3-Smad4 site identified as described above (Fig. 4) remained intact. This suggests that a synergistic functional cooperation exists between Smads and AP-1/CRE complexes in the context of TGF-β-induced transcriptional activation of this c-Jun promoter region.

We next sought to identify which proteins bind to this AP-1/CRE site in our system. We performed an EMSA using the −79 to −19 probe diagrammed in Fig. 2, where we had observed that there was no change in the pattern of binding to this sequence upon TGF-β addition. Since previous studies (15) had identified c-Jun and ATF-2 as the components constitutively bound to this site, we attempted to supershift the bound complexes with antibodies against these two transcrip-

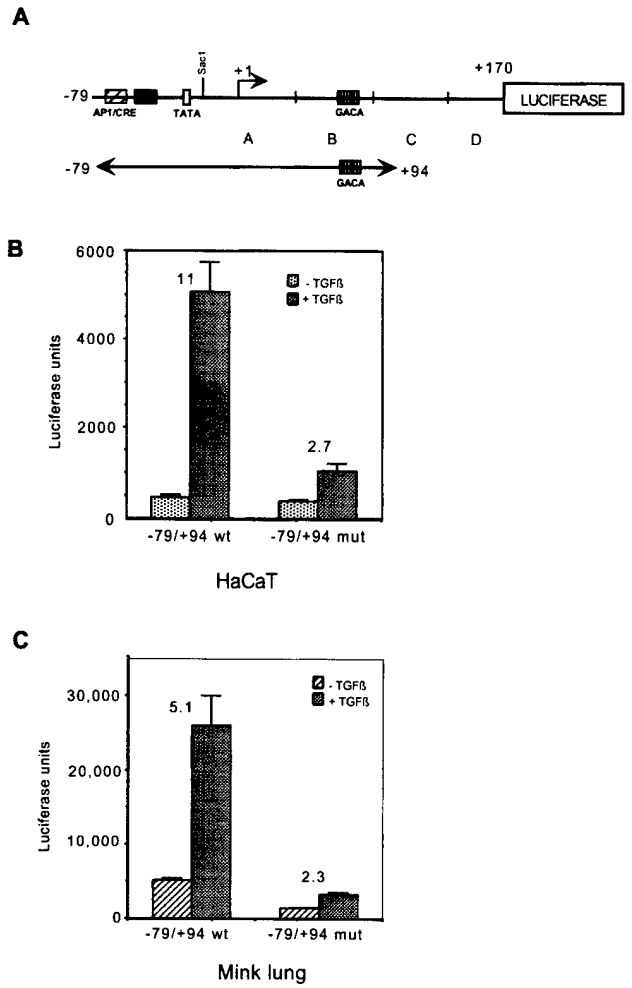

FIG. 4. Mutation of the Smad3-Smad4 binding site abrogates responsiveness to TGF-β. (A) Diagram of new reporter constructs created by PCR mutagenesis. Two reporters for the region from −79 to +94 of the c-Jun promoter were created, i.e., one with wild-type sequence and the other mutated at the Smad3-Smad4 binding site (CAGA triplet) from +62 to +73. (B) The −79 to +94 wild-type and −79 to +94 mutant reporters were transfected into HaCaT cells. Cells were treated with TGF-β1 for 24 h before harvesting for luciferase assays. Fold inductions were calculated by comparing the luciferase activities of TGF-β-treated cells and untreated control cells. (C) The procedures used were the same as described for panel B except that Mv1Lu cells were used instead of HaCaT cells.

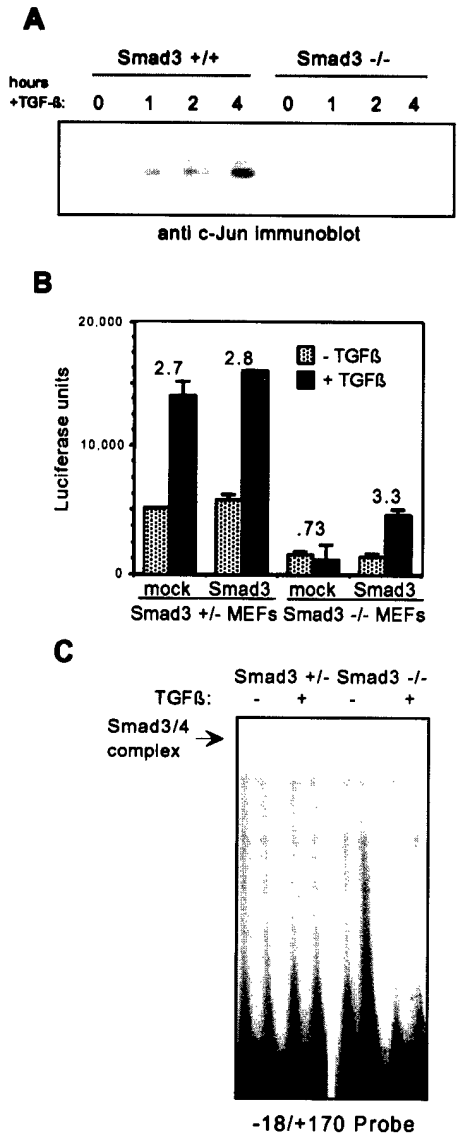

FIG. 5. Induction of c-Jun by TGF-β is lost in Smad3 null fibroblasts. (A) Western blotting was performed by using nuclear lysates from $Smad3^{+/+}$ or $Smad3^{-/-}$ primary MEFs treated with TGF-β1 for 0, 1, 2, or 4 h. MEFs were serum starved for 12 h in 0.2% serum before treatment. (B) The −79 to +170 reporter was transfected into $Smad3^{+/-}$ or $Smad3^{-/-}$ MEFs with empty expression vector (mock) or Smad3 expression vector (Smad3). Cells were treated with TGF-β1 for 24 h before harvesting for luciferase assays. Fold induction by TGF-β1 is indicated over the bars. (C) EMSA was performed by using the −18 to +170 probe and nuclear lysates from untreated $Smad3^{+/-}$ MEFs or $Smad3^{-/-}$ MEFs or cells of the same types treated with TGF-β1 for 1 h. The induced Smad3-Smad4 complex is indicated with an arrow.

tion factors. An antibody specific to c-Jun caused a supershift of the slower-migrating complex, confirming the presence of c-Jun (Fig. 6B). However, we did not see a supershift on this probe when we used two commercially available antibodies specific to ATF-2 (Fig. 6B), which had been successfully used to supershift ATF-2-containing complexes in a previous study (11). Additionally, several commercial antibodies against CREB were unable to supershift this complex (data not shown). Nonetheless, we were able to compete away binding of the faster- and slower-migrating complexes using unlabeled consensus CRE site oligonucleotide in 200× molar excess, whereas the same molar excess of unlabeled mutant CRE oligonucleotides did not compete with the binding. This suggests that a component of the bound complexes is a CRE binding protein. These results demonstrate that a constitutively bound complex containing c-Jun, either as a homodimer or in combination with a yet unknown CRE binding partner, is required in conjunction with the Smad complex in mediating the TGF-β activation of this promoter region.

DISCUSSION

We identify here a novel Smad3-Smad4 binding site in the 5′ untranslated region (UTR) of the c-Jun promoter and introduce evidence for the simultaneous requirement for two different responsive elements in mediating TGF-β-induced c-Jun transcription. The first is a Smad3-Smad4 binding site, and the second is a spatially distinct AP-1/CRE binding site. The two elements are capable of binding their corresponding transcrip-

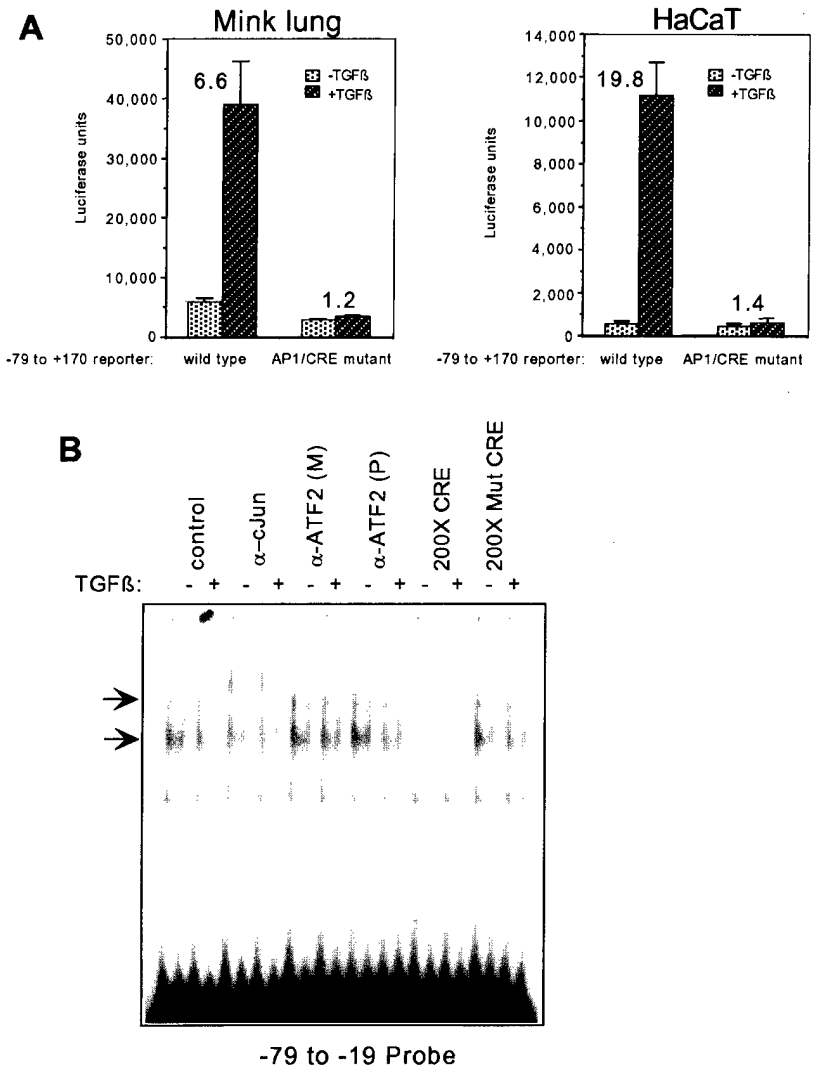

FIG. 6. An AP-1/CRE site is also required for TGF-β and Smad responsiveness of the c-Jun promoter. (A) A −79 to +170 luciferase reporter carrying a mutation in the AP-1/CRE site induced by PCR mutagenesis was transfected into HaCaT cells alongside the wild type −79 to +170 reporter. The mutation changed the sequence from TGACATCA to ATCCACCA. Fold induction was calculated by comparing TGF-β-treated cells to untreated control cells. Cells were treated with TGF-β1 for 24 h before harvesting for luciferase assays. (B) EMSA was performed as described in the legend for Fig. 2B. Attempts to perform supershifts were made using a monoclonal antibody against c-Jun (α-cJun) and polyclonal [α-ATF2(P)] and monoclonal [α-ATF2(M)] antibodies against ATF-2 (third through eighth lanes). Competition with 200-fold molar excess of wild-type CRE consensus site oligonucleotide (200× CRE) or 200-fold molar excess of mutated CRE site oligonucleotide (200× Mut CRE) is illustrated in the last four lanes.

tion factor complexes independently. Importantly, mutation of either element alone severely diminishes TGF-β responsiveness, suggesting that the two elements have a functionally synergistic relationship. This notion is supported by the fact that, in an additive system, mutation of either element would result in only a partial loss of TGF-β response; a complete loss of TGF-β response would require the simultaneous mutation of all contributing elements because each element could function alone to mediate a partial response. On the other hand, in a synergistic relationship such as the one we have identified, neither element is capable of mediating a vigorous transcriptional response in the absence of the other, so the effect of the two elements acting together is greater than the sum of the effects of each element alone.

These findings introduce important nuances into the developing model of Smad-mediated transcriptional regulation and offer an illustration to support aspects of Smad function predicted by biochemical and structural observations. They suggest that synergy between Smads and other transcription factors could be an important mechanism for mediating both the specificity and the responsiveness to cross talk of the TGF-β transcriptional activation signal.

Sequence comparison of Smad3-Smad4 binding sites. Numerous studies have identified Smad3-Smad4 DNA binding sites using various approaches. As seen in Table 1, the sequences found by various groups are essentially identical; regardless of whether one defines a Smad3-Smad4 binding site as the palindrome AGACGTCT, as the CAGA box, or as repeats of GACA, all of the identified sites contain the Smad box, 5′-GTCT-3′, or its reverse complement, 5′-AGAC-3′ (9, 57, 58). Most recently, another Smad3-Smad4-responsive site, CAGACAGtCTGTCTG in the *junB* promoter, was identified (18). Only the COL7A1 promoter presents a discrepancy, in that the deletions which abrogate Smad binding do not directly

TABLE 1. Comparison of Smad3-Smad4 binding sites

Reference	Location	Sequence
This study	c-Jun, +62 to +73	CAGACAGACAGACACA
9	PAI-1	
	−730	AGCCAGACA
	−580	AGACAGACA
	−280	AGACAGACA
58	Oligo screen	GTCTAGAC
57	2X TRE (from p3TP-lux)	TGAGTCAGACA (21 bp) TGAGTCAGACA

disturb the Smad box-like sequences (54). It may be, as the authors suggest, that the small deletions at the ends of their binding element disrupted binding in a non-sequence-specific manner.

In agreement with these other studies, the novel Smad3-Smad4 binding site identified in the c-Jun 5′ UTR consists of three Smad boxes in a row. Although it is unusual to find enhancing elements in the 5′ UTR, it is not unprecedented. Transcriptional activators with binding sites in the 5′ UTR of promoters or in intronic sequences are hypothesized to function transiently, i.e., during the establishment of the initiation of transcription (5, 32, 44). The molecular mechanism for Smad-mediated activation of transcription is not yet well defined, but a transient role of Smads in transcriptional initiation, through their binding to the sequence in the 5′ UTR of the c-Jun gene, would be consistent with the transient presence of Smads in the nucleus after TGF-β stimulation.

The Smad consensus binding site, or Smad box, has now been confirmed by the elucidation of the crystal structure of Smad3 bound to DNA (46). A single Smad3 MH1 binds asymmetrically through a novel DNA binding β-hairpin structure to a 4-bp Smad box (CAGA) with sequence-specific interactions (9, 58). Note that in vivo Smads exist as homo- and hetero-oligomers (20, 25, 46, 56, 61), which would explain why more than one 4-bp repeat has been found to be required for binding of natural Smad complexes in the studies discussed above.

Synergy between Smads and AP-1 family members. Our results further demonstrate that while the Smad3-Smad4 site is important for TGF-β induction of c-Jun, an AP-1/CRE site is also required for TGF-β regulation of the c-Jun promoter. Mutation of either site in the context of the −79 to +170 region of this promoter eliminated the ability of TGF-β to elicit maximal induction of the c-Jun promoter. There are several possible mechanisms by which such synergy may be achieved, and elucidating the mechanism for this synergistic cooperation is an important area for future investigations.

The first possibility is that direct physical interaction between Smads and AP-1 family members is responsible for mediating the functional cooperation. Recent studies have described an interaction between Jun family members and Smad3 (27, 60). In fact, Smad3 and Smad4 have both been found to interact with all members of the Jun family to varying degrees. The Jun family members interact with Smads at a small C-terminal domain which is highly conserved among Jun proteins. While the interaction between Jun and Smads is direct, the involvement of this protein-protein interaction in transcriptional activation of the c-Jun promoter is unclear. Although it is a strong possibility, direct protein interaction is certainly not the only possible explanation for the observed functional cooperation seen in the c-Jun promoter between Smads and AP-1.

Another possible mechanism for functional synergy is cooperative DNA binding. We do not know whether AP-1/CRE complexes and Smad3-Smad4 complexes may cooperatively bind their corresponding sites in vivo, even though they clearly can strongly bind their corresponding c-Jun promoter sites independently in vitro. It is possible that the interactions of each complex with DNA in vivo may be enhanced by cooperative recruitment and stabilization or by an alteration in local DNA structure which is fostered by the binding of both complexes at once.

Synergy is a functional cooperation that can also be independent of any physical interaction. It is possible that Smads and AP-1 may cooperate by contributing complementary but necessary subfunctions of transcriptional activation, for instance by recruiting different required members of the basal transcriptional machinery. The location of the Smad binding site 3′ in relation to the TATA box in the c-Jun promoter strongly suggests that the role of Smads is transient and limited to the start of transcription, perhaps involving the establishment of the transcription initiation complex. AP-1 may contribute complementary functions to promote transcription.

Finally, it is possible that TGF-β signal transduction can directly affect the activity of AP-1 complex bound to the promoter element. It has been postulated that TGF-β may signal through the mitogen-activated protein kinase pathway and activate AP-1 through phosphorylation. This remains to be clearly shown and is currently under investigation. Such an activity would add yet another dimension to the cooperativity in the c-Jun promoter region demonstrated here.

Further implications. The model of required synergistic cooperation may explain some discrepancies in our understanding of Smad function to date. A number of recent studies have established the abilities of Smad3 and Smad4 to interact and function synergistically with the transcriptional coactivator CREB binding protein/p300 (10, 17, 40, 45, 52). Although these findings suggest that a DNA-bound Smad3-Smad4 complex is able to independently recruit CREB binding protein/p300 and hence possibly initiate transcription on its own, it does not appear to do so. A close examination of studies on Smad3-Smad4 binding sites reveals that no single Smad3-Smad4 site has been found to be sufficient for TGF-β responsiveness. In all of these studies, multiple copies of the Smad binding site were found to be required to confer TGF-β responsiveness (9, 58). Perhaps a single Smad3-Smad4 complex is unable to successfully recruit the factors necessary to accomplish transcriptional activation on its own. Cooperation with another transcription factor, such as AP-1, Sp1 (33), or TFE3 (16), or collaboration between a number of Smad3-Smad4 binding sites is required to build strong enough interactions to activate transcription.

It is worth noting that while we have examined responsive

elements and Smad binding in the −79 to +170 region of the c-Jun promoter, there may be additional Smad3-Smad4 binding sites, or other TGF-β responsive elements, elsewhere in the native c-Jun promoter sequence. These could in fact cooperate further with the Smad3-Smad4 binding site identified in this study to mediate c-Jun regulation in vivo.

Finally, an investigation into other examples of cooperating responsive elements could yield critical insight into TGF-β signaling specificity and cross talk with other signaling pathways. Given the description in the present study of a joint requirement for Smad3-Smad4 binding and an AP-1/CRE site, it may be interesting to look for additional required elements in other TGF-β-responsive promoters. The recent work by Hua et al. (16) revealed another important example of such cooperativity and lends further support to the possibility that similar modes of synergistic transcriptional activation may exist in the context of many Smad-responsive promoters.

We have identified in these studies a functional cooperation between a novel Smad3-Smad4 site and an AP-1/CRE binding site within the −79 to +170 region of the c-Jun promoter, which functions in transcriptional activation by TGF-β. These findings not only solidify the role of Smad3 as an intracellular effector for the TGF-β signal but also support a new and more complex model of Smad3-Smad4 transcriptional regulation, i.e., one which involves cooperation with neighboring response elements and may allow coordination of other interacting pathways with the TGF-β signal. The synergistic interaction between TGF-β-specific effectors and other transcription factors proposed in this model could mediate the activation of different subsets of target genes in different cell types and physiological states, translating into the diversity of physiological and pathological roles played by TGF-β in different tissue types, stages of development, and disease states.

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C.W. and E.M.R.-C. contributed equally to this work.

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Biochemistry

Smads bind directly to the Jun family of AP-1 transcription factors

(Smad3/Smad4/cJun/JunB/transforming growth factor)

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ABSTRACT **Smad3 and Smad4 are sequence-specific DNA-binding factors that bind to their consensus DNA-binding sites in response to transforming growth factor β (TGFβ) and activate transcription. Recent evidence implicates Smad3 and Smad4 in the transcriptional activation of consensus AP-1 DNA-binding sites that do not interact with Smads directly. Here, we report that Smad3 and Smad4 can physically interact with AP-1 family members. *In vitro* binding studies demonstrate that both Smad3 and Smad4 bind all three Jun family members: JunB, cJun, and JunD. The Smad interacting region of JunB maps to a C-terminal 20-amino acid sequence that is partially conserved in cJun and JunD. We show that Smad3 and Smad4 also associate with an endogenous form of cJun that is rapidly phosphorylated in response to TGFβ. Providing evidence for the importance of this interaction between Smad and Jun proteins, we demonstrate that Smad3 is required for the activation of concatamerized AP-1 sites in a reporter construct that has previously been characterized as unable to bind Smad proteins directly. Together, these data suggest that TGFβ-mediated transcriptional activation through AP-1 sites may involve a regulated interaction between Smads and AP-1 transcription factors.**

Transforming growth factor β (TGFβ) is a multipotent cytokine that regulates a variety of cellular activities, such as cell proliferation, differentiation, and extracellular matrix (ECM) formation. The combined actions of these cellular responses are likely to mediate more global effects of TGFβ including its role in development, wound healing, immune responses, and the pathogenesis of cancer (1–3). The identification of genes transcriptionally regulated by TGFβ and the elucidation of the molecular mechanisms responsible for this transcriptional regulation will help define how TGFβ exerts its cellular effects and its role in resulting physiological processes. Although progress has been made in the identification of TGFβ target genes, including the cyclin-dependent kinase inhibitors p21 and p15 (1, 2) and the ECM component plasminogen activator inhibitor-1 (PAI-1) (3), which has subsequently contributed toward our understanding of TGFβ-mediated growth inhibition and ECM deposition, the mechanisms by which TGFβ controls gene expression remain largely unknown.

Numerous studies have characterized the differential expression of specific genes in response to TGFβ, revealing a common link in the ability of TGFβ to regulate many of these genes through the functions of the AP-1 family of transcription factors. This protein family, which includes the Fos and Jun proteins, binds a specific DNA sequence and facilitates transcriptional regulation (4). The ability of TGFβ to induce the expression of several genes, including PAI-1, clusterin, monocyte chemoattractant protein-1 (JE/MCP-1), type I collagen, and TGFβ1 itself depends on specific AP-1 DNA-binding sites in the promoter regions of these genes (3, 5–10). Furthermore, TGFβ-mediated transcriptional activation of several of these genes requires AP-1 proteins (5, 8–10). Intriguingly, the expression of many AP-1 proteins themselves is induced as an early response to TGFβ in a cell type-specific manner (11–14). It has been demonstrated that this induced expression of particular AP-1 family members is involved in TGFβ-mediated regulation of subsequent target genes (10). In addition, genetic studies of TGFβ signaling in *Drosophila melanogaster* reveal a direct overlap between AP-1 and TGFβ signaling and suggest an evolutionarily conserved convergence of these pathways (15). Together, these studies demonstrate a link between TGFβ signaling and AP-1 in the TGFβ-regulated expression of various genes. The molecular mechanisms responsible for the TGFβ-mediated transcriptional activation of these genes are just beginning to be elucidated.

Insight into the mechanism of TGFβ-regulated gene expression has come about with the discovery of the Smad family of proteins. The Smads are phosphorylated by the activated type I receptor in response to ligand (16). Specifically, Smad2 and Smad3 were shown to be inducibly phosphorylated in response to TGFβ (17–19). Smad phosphorylation results in heteromerization of either Smad2 or Smad3 with Smad4 (20–23). Smad4-containing heteromers then enter the nucleus where they can activate transcription of specific genes (24, 25). Current research is focused on elucidating the role of Smads in TGFβ-induced transcriptional activation.

Through attempts made at understanding the mechanism of Smad-mediated transcriptional activation, two distinct roles for Smads have emerged: Smads as DNA-binding factors and Smads as transcription factor-binding proteins. Several lines of evidence suggest that Smads activate transcription by binding directly to DNA. For instance, transcription of a reporter plasmid containing the concatamerized consensus Smad-binding site is induced by TGFβ in a Smad4-dependent manner (26). Smad3 and Smad4 were recently shown to form a complex on similar DNA sequences derived from the PAI-1 promoter (27). Mutation of these sequences in the PAI-1 promoter reduced TGFβ responsiveness. Furthermore, Gal4 fusions with the C-terminal domains of Smad1 and Smad4 activate transcription from concatamerized Gal4 DNA-binding sites (28).

Other evidence suggests that Smads can activate transcription by binding to other transcription factors. For example, the interaction between Smad2/Smad4 heteromers and the transcription factor FAST-1 is critical for the formation of the

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Abbreviations: TGFβ, transforming growth factor β; HaCaT, human keratinocyte cells; JNK, cJun N-terminal kinase; CMV, cytomegalovirus; GST, glutathione *S*-transferase; TNT, transcription and translation.

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activin responsive factor (ARF), an activin-inducible DNA-binding complex in *Xenopus* (24, 29). Overexpression of the Smad-binding domain of FAST-1 blocked ARF formation and transcriptional induction of an activin-inducible early response gene. Together, these data indicate that although Smads bind DNA directly, association with other transcription factors may play a crucial role in Smad-mediated transcriptional activation.

In an attempt to identify transcription factors involved in Smad-mediated transcriptional activation, we performed a yeast two-hybrid screen using Smad3 as a bait. Two interacting cDNAs encoding two different clones of the AP-1 family member, JunB, were isolated, indicating that Smads may bind to AP-1 members directly. Supporting a direct interaction between Smads and AP-1, we show that Smad3 and Smad4 bind all known members of the Jun family of proteins *in vitro*. Furthermore, we demonstrate that Smad3 is critical for the ability of TGFβ to activate AP-1 sites independent of Smad DNA binding. These data, therefore, provide insight into a possible mechanism by which TGFβ activates AP-1-mediated transcription through the induction of Smad/AP-1 complex formation.

MATERIALS AND METHODS

Materials. TGFβ1 was a generous gift of Amgen Biologicals. Human keratinocyte cells (HaCaT) were the generous gift of P. Baukamp and N. Fusenig. A HaCaT cDNA library in the pACT2 expression vector was the generous gift of Y. Xiong. The full length cDNAs for murine Jun family members, FosB, cFos, Fra2, and human Fra1, were the generous gifts of R. Wisdom. Smad3 polyclonal antibody was generated against amino acids 200–219 of Smad3 and affinity purified in this laboratory. Antibodies used included JunB polyclonal N-17 (Santa Cruz Biotechnology), cJun monoclonal KM-1 (Santa Cruz Biotechnology), cJun polyclonal no. 9162 (NEB, Beverly, MA), cJun polyclonal no. 06–828 (Upstate Biotechnology, Lake Placid, NY) and JunD-329 polyclonal antibody (Santa Cruz Biotechnology).

Cell Culture. COS cells were maintained in DMEM with 10% FBS. HaCaT cells were maintained in MEM with 10% FBS. Primary fibroblasts were prepared from day 14 embryos by mechanical dissociation of whole embryos by passage through an 18-gauge needle and plating onto gelatin-coated 10-cm tissue culture plates in DMEM with the inclusion of 20% FBS. Cells were grown to confluence and carried in DMEM with 10% FBS. All experiments were performed on littermate fibroblasts at the same passage number.

Plasmid Construction. The *Bam*HI fragment containing full length human Smad3 cDNA was subcloned from pGEX-3X into pGBT9 (CLONTECH) (30). cDNAs encoding each Jun and Fos family member were subcloned into pCMV5 and pCMV6 expression vectors (CMV, cytomegalovirus). Full length JunB was PCR amplified with the following primers: CGGGATCCCGATGTGCACGAAAATGG (5′ primer) and GGATCCTCAGAAGGCGTGTCC (3′ primer). Full length cJun was PCR amplified with the following primers: CGGGATCCCGATGACTGCAAAGATGGAAACG (5′ primer) and CGGGATCCCGTCAAAACGTTTGCAACTGC (3′ primer). The cDNAs were completely sequenced and subcloned into pACT2 (CLONTECH). Construction of 4xSBSMT and 4xAP1MT reporter plasmids was previously described (30).

Yeast Two-Hybrid Assay. The yeast strain Hf7c was transformed with Smad3/pGBT9, and expression of the appropriate-size fusion protein was confirmed by Western blotting by using GAL4 DBD monoclonal antibody (Santa Cruz Biotechnology). Bait-expressing yeast were transformed with a HaCaT cDNA library in the pACT2 expression vector. Individual cDNAs (5×10^6) were screened. Transformants (484) grew on media lacking tryptophan, leucine, and histidine that contained 5 mM 3-aminotriazole. Transformants (242) were positive for β-galactosidase activity, which was measured by the appearance of blue color on colony filter lifts incubated in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). Bait dependence for each positive transformant was established similarly.

Binding Studies. Full length JunB in pGEM4 was digested with *Bsp*HI, BssHII, or *Dra*I (NEB). The full length construct and the digested DNAs were used as templates for *in vitro* transcription and translation (TNT) with [^{35}S]methionine in rabbit reticulocyte lysates (Promega). The TNT–JunB lysates were incubated with an equal amount of bacterially purified glutathione *S*-transferase (GST), or GST-Smad3 or GST-Smad4 (30) in B/P (150 mM NaCl/50 mM Tris, pH 7.5/0.1% Tween/1 mM DTT) for 2.5 hours at 4°C. The GST reactions were washed three times in TBS (500 mM NaCl/25 mM Tris, pH 7.5/0.1% Tween-20/1 mM DTT). Samples were resolved by SDS/PAGE. The gels were treated with 10% sodium salicylate, dried, and exposed to film. Whole-cell COS lysates overexpressing each AP-1 member were lysed as described (30) and incubated with the GST fusions as described above. The binding reactions were washed three times with B/P and separated by SDS/PAGE.

For endogenous protein interactions, HaCaT cells were treated with 100 pM TGFβ1 in DMEM/10% FBS for 15, 30, or 60 min. Cells were then lysed and either whole-cell or nuclear extracts were prepared (30, 31). Four hundred fifty μg of each whole-cell lysate was incubated with an equal amount of bacterially purified GST-Smad3 or GST-Smad4 normalized for protein by Coomassie blue and for volume of glutathione-Sepharose added to each binding reaction. After 2 hr at 4°C, the reactions were washed three times and separated by SDS/PAGE. One hundred μg of each nuclear lysate was diluted to 150 mM NaCl with buffer A and incubated with GST, GST-Smad3, or GST-Smad4, as above. For phosphatase treatment, HaCaT cells were treated for 15 min with 100 pM TGFβ1 or DMEM containing 10% FBS, penicillin, streptomycin, and 0.5 M sorbitol and whole-cell extracts were prepared as described (30). Briefly, the lysates were treated with potato acid phosphatase (0.034 units) and calf intestinal phosphatase (2 units) for 30 min at 37°C.

Western Blot Analysis. Electrophoresed proteins were transferred to Immobilon (Millipore) and treated as previously described, except that the blots were blocked and blotted in PBS/0.1% Tween-20/5% milk (30).

Luciferase Assays. Transfections were performed by using a standard DEAE-Dextran protocol (32). Primary fibroblasts were allowed to recover from glycerol shock for 20 hr before treating with 100 pM TGFβ1 in DMEM/0.2% FBS. Luciferase assays were performed as previously described (33). All transfections were normalized to β-galactosidase activity by co-transfection of 0.5 μg of CMV-β-galactosidase expression vector.

RESULTS

Smad3 and JunB Associate in Yeast. To identify Smad3-binding proteins, we performed a yeast two-hybrid screen using the Gal4 DNA-binding domain fused to Smad3 as a bait. Of the five million yeast transformants screened for Smad3 binding, 242 transformants were positive for growth in the absence of histidine and for the appearance of blue color on staining with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal). Two of the clones sequenced contained two different cDNA fragments encoding the AP-1 member, JunB (Fig. 1). Clone 44 lacked the N-terminal 126 amino acids of JunB indicating that these residues are not required for binding in yeast. Fusions of the Gal4 activation domain with cDNAs encoding JunB and cJun also tested positive for interaction with the Smad3 bait protein (data not shown).

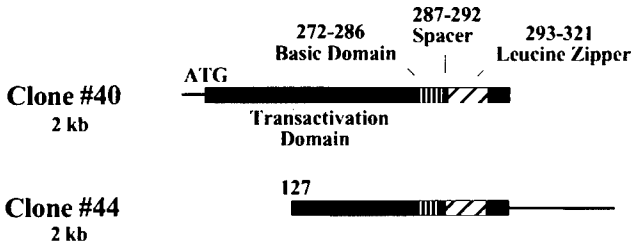

FIG. 1. Isolation of JunB from a Gal4-Smad3 screen of a human keratinocyte library (HaCaT) in yeast. Schematic diagram of two Smad3-interacting clones encoding JunB. Amino acids associated with particular functional domains of JunB are shown.

***In Vitro* Binding of Smads and AP-1.** To determine whether this interaction occurs in solution with recombinant proteins, GST pulldown experiments were performed. Bacterially produced GST-Smad3, but not GST alone, bound TNT-JunB (Fig. 2*B*). In addition, GST-Smad4-bound TNT–JunB, indicating that AP-1 binding is not exclusive to Smad3. Similar studies with GST-Smad1, GST-Smad2, and GST-Smad5 showed that these proteins also bind JunB TNT products but with lower affinity than that observed with GST-Smad3 and GST-Smad4 (data not shown). To map the Smad interaction domain, GST-Smad fusion proteins were used to pull down various TNT–JunB deletion products (Fig. 2*A* and *B*). Deletion of only 20 amino acids from the C terminus of JunB abrogated the interaction between GST-Smad3 and GST-Smad4 and JunB (Fig. 2). Thirteen of these 20 amino acids are conserved among Jun family members, including cJun and JunD (Fig. 3*A*). To

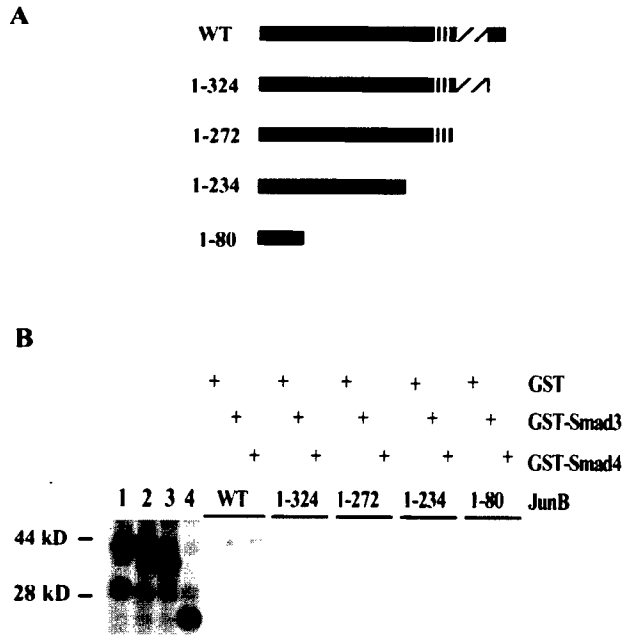

FIG. 2. *In vitro* association of Smad3 and Smad4 with JunB. (*A*) Schematic diagram of JunB deletion mutants used in the binding study shown in *B*. (*B*) *In vitro* association of JunB and JunB deletion mutant TNT products with GST, GST-Smad3, or GST-Smad4. Bacterially produced GST, GST-Smad3, and GST-Smad4 proteins were coupled to glutathione-Sepharose, incubated with the indicated TNT product, centrifuged, and the Sepharose-bound proteins visualized by Coomassie stained SDS/PAGE (data not shown). The amount of each GST protein and the volume of glutathione-Sepharose used in each binding reaction were normalized. Each reticulocyte lysate was produced as described in *Materials and Methods*. Domains depicted in *A* refer to the same domains depicted in Fig. 1. Five percent of each reticulocyte lysate input was run in lanes 1–4: lane 1, JunB full length; lane 2, 1–324; lane 3, 1–272; lane 4, 1–80.

determine whether these proteins also interact with Smads, we tested whether GST-Smad3 and GST-Smad4 could associate with AP-1 members from transfected COS cell lysates overexpressing each Jun member. As shown in Fig. 3*B*, all three Jun family members associated with both GST-Smad3 and GST-Smad4 to a similar extent. Furthermore, GST-Smad3 and GST-Smad4 bound full length TNT–cJun and deletion of 20 amino acids from the C terminus of TNT–cJun also reduced association with GST-Smad3 and GST-Smad4 (data not shown). Conversely, in studies using lysates from transfected cells overexpressing each Fos family member, no association with GST-Smad3 or GST-Smad4 was observed (data not shown). Consistent with these findings, the amino acids required for Jun binding to GST-Smads *in vitro* are not conserved among Fos family members.

Smad3 and Smad4 Associate with An Inducibly Phosphorylated Form of Endogenous cJun. To determine whether GST-Smad3 and GST-Smad4 can associate with endogenous cJun, we incubated GST-Smad3 and GST-Smad4 with TGFβ-treated HaCaT whole-cell extracts. Western blot analysis of these binding reactions with a cJun-specific antibody raised against a phosphopeptide containing phosphorylated Ser-63 of cJun (KM-1 from Santa Cruz Biotechnology) showed that GST-Smad3 and GST-Smad4 bind this form of cJun (Fig. 4*A*). We noticed that the level of cJun recognized by the KM-1 antibody appeared to increase with TGFβ treatment. To more clearly determine whether the level of cJun was induced by TGFβ, nuclear lysates were analyzed by using the KM-1 antibody and a different cJun antibody raised against a nonphosphorylated N-terminal portion of cJun (no. 9162, NEB). As shown in Fig. 4*B*, the form of cJun recognized by the KM-1 antibody is clearly induced by TGFβ treatment, whereas the levels of total cJun recognized by the NEB antibody remain relatively unchanged. This result suggests that cJun is rapidly phosphorylated, at least on the residue of Ser-63, in response to TGFβ. Subsequent binding studies with these nuclear lysates and GST, GST-Smad3, and GST-Smad4 revealed that GST-Smad3 and GST-Smad4 bound equally well to cJun recognized by either antibody (data not shown). To confirm that the TGFβ-induced increase in the cJun species detected by the KM-1 antibody was indeed the phosphorylated form of cJun, we performed a phosphatase assay using HaCaT lysates treated with TGFβ (Fig. 4*C*). As a positive control, HaCaT cells were treated with 0.5M sorbitol, which has been shown to induce cJun N-terminal kinase (JNK) kinase activity (34), and similar results were obtained with lysates treated with 50 μg/ml anisomycin. Under phosphatase treatment conditions, the TGFβ-induced form of cJun recognized by the KM-1 antibody was lost, whereas total cJun detected by the no. 9162 antibody did not change. The specificity of the KM-1 antibody for the phosphorylated form of cJun was further confirmed by using another antibody (no. 06–828, Upstate Biotechnology) raised against a cJun peptide also containing phosphorylated Ser-63.

Smad3 Is Required for TGFβ-Mediated Activation of AP-1 Sites Independent of Smad DNA-Binding Activity. In an attempt to determine whether the interaction between AP-1 and Smads contributes to the ability of TGFβ to activate the transcription of AP-1 sites, we transfected Smad3 heterozygous and Smad3 homozygous null primary mouse embryonic fibroblasts with AP-1 site-containing reporter constructs previously described (Fig. 5) (30). Intriguingly, 4xSBSMT, which contains consensus AP-1 sites adjacent to mutated Smad DNA-binding sites incapable of Smad protein binding, requires Smad3 for transcriptional activation by TGFβ. TGFβ is unable to activate transcription, however, in the analogous reporter, 4xAP1MT, in which the Smad-binding sites are intact and the AP-1 sites are mutated. Thus, within the context of the 4xSBSMT reporter, the AP-1 sites are required for transcriptional activation by TGFβ. Activation of these AP-1 sites

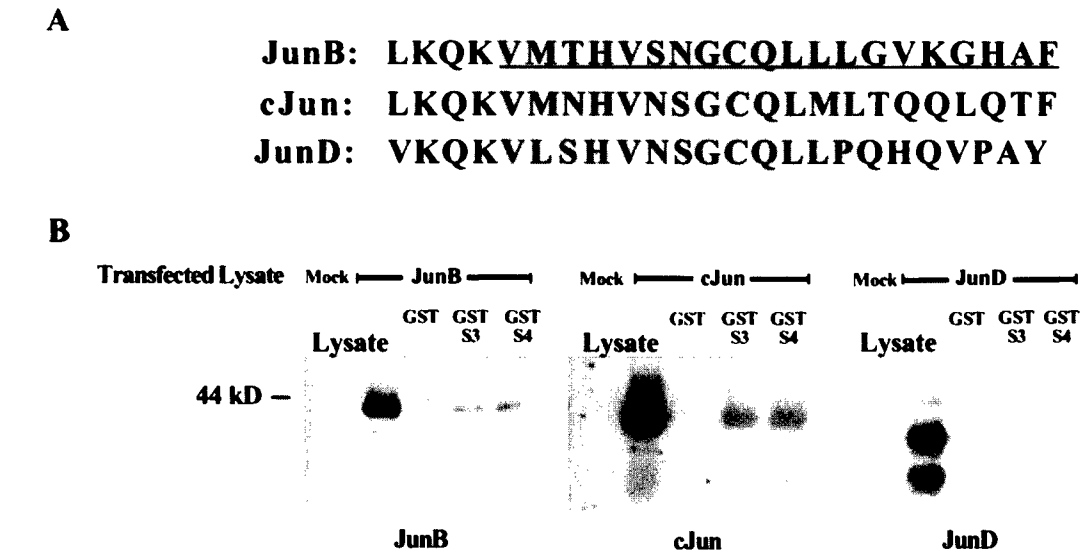

FIG. 3. Association of Smad3 and Smad4 with Jun family members. (*A*) The Smad-binding site on JunB is conserved among Jun family members. The very C-terminal amino acids of the Jun proteins are aligned. The leucine at the beginning of the JunB and cJun sequences is the most C-terminal leucine of the leucine zipper domain. Amino acids that were deleted in the JunB 1–324 mutant are underlined. Conserved amino acids that may play a role in Smad binding are shaded. (*B*) *In vitro* association of overexpressed JunB, cJun, and JunD from COS cell lysates with GST, GST-Smad3, or GST-Smad4. COS cells transfected with JunB/pCMV, cJun/pCMV, or JunD/pCMV were lysed, and these extracts were treated with GST fusion proteins prepared as described in *Materials and Methods.*

requires Smad3 but is independent of Smad DNA binding, suggesting that the ability of Smad3 to act through the AP-1 sites in this reporter is required for transcriptional activation by TGFβ.

DISCUSSION

Over the past year, a model for the functional role of Smads in TGFβ-mediated transcriptional regulation has emerged. Here, we provide evidence supporting a role for Smads as transcriptional coactivators, in addition to their role as DNA binding-dependent activators of transcription. Smads may thus transduce the TGFβ signal to the promoter level and activate transcription through direct physical interaction with DNA-bound AP-1 proteins.

The potential role of Smads as transcriptional coactivators of AP-1 is supported by a previous study in which we reported that TGFβ as well as Smad3/Smad4 cooverexpression could activate transcription of 4xWT, a luciferase reporter containing a concatamerized TGFβ-responsive element derived from

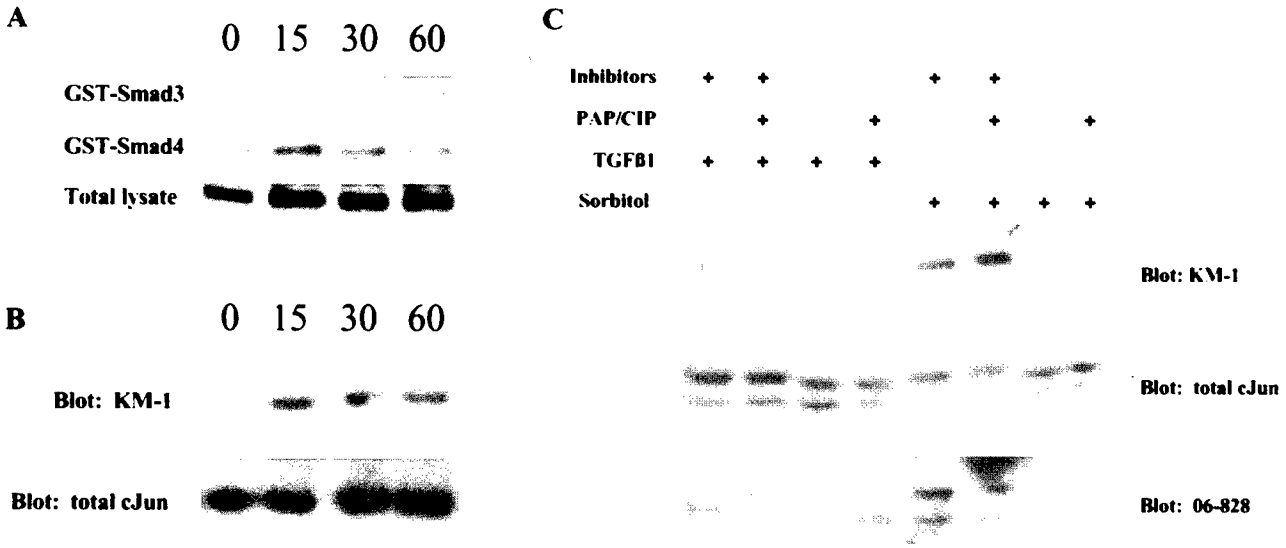

FIG. 4. GST-Smad3 and GST-Smad4 associate with an inducibly phosphorylated form of endogenous cJun. (*A*) HaCaT cells were treated with 100 pM TGFβ1 for 15, 30, or 60 min and lysed. GST-Smad3 and GST-Smad4 were incubated with 450 μg of whole-cell HaCaT lysates, and the binding reactions were analyzed by Western blot by using a cJun specific monoclonal antibody raised against a phosphopeptide containing phosphorylated Ser-63 of cJun, KM-1 from Santa Cruz. The lower row shows 60 μg total lysate blotted with the same antibody as the upper rows. (*B*) Nuclear lysates (60 μg) from HaCaT cells treated as in *A* were analyzed with the KM-1 antibody. This blot was stripped and reprobed with antibody no. 9162 from NEB raised against a fusion protein containing the unphosphorylated N-terminal portion of cJun. (*C*) HaCaT cells were treated with 100 pM TGFβ1 or 0.5 M sorbitol for 15 min, lysed in the absence or presence of phosphatase inhibitors, and treated with or without potato acid phosphatase and calf intestinal phosphatase. The reactions were analyzed by SDS/PAGE and Western blotting. The upper row shows 60 μg of each lysate blotted with the KM-1 antibody. This blot was stripped and reprobed with no. 9162 antibody, shown in the middle row. This blot was stripped again and reprobed with no. 06–828 antibody (Upstate Biotechnology). The bands in the KM-1 blot align with the upper bands in the no. 9162 antibody and the no. 06–828 antibody blots.

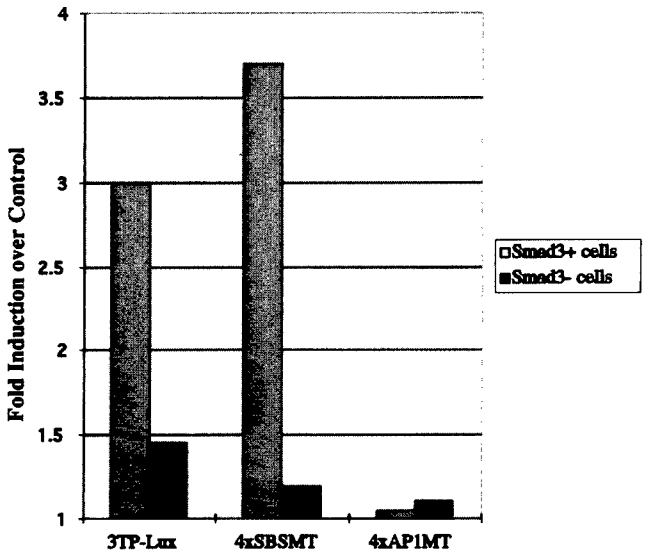

FIG. 5. Smad3 is required for TGFβ-mediated transcriptional activation of 4xSBSMT. Smad3 homozygous null or Smad3 heterozygous mouse primary fibroblasts were transfected with 3 μg of 3TP-Lux, 4xSBSMT, or 4xAP1MT reporter constructs. Twenty hours after transfection, vehicle or 100 pM TGFβ1 was added. Twenty-four hours after treatment, luciferase activity was quantified. The averages of duplicate transfections were used to determine the fold induction by TGFβ over the vehicle control. The 3TP-Lux data is shown as a positive control for the experiment (35).

3TP-Lux that contains both consensus AP-1 binding sites and Smad4-binding sites (30). Interestingly, a mutation in this sequence that inhibited Smad association had no effect on the ability of TGFβ or Smad3/Smad4 cooverexpression to induce transcription of this reporter. In contrast, a mutation in the adjacent AP-1 site that inhibited the association of a constitutively bound, AP-1-containing complex, abrogated the ability of TGFβ or Smad overexpression to activate transcription of the 4xWT reporter. These data suggest that within the context of this reporter, TGFβ-mediated transcriptional activation does not depend on the DNA- binding function of Smads but rather the ability of Smads to act through the AP-1 sites. In light of the interaction studies described here, Smads may act through these AP-1 sites by binding directly to AP-1 proteins. In studies of Smad3 wild-type and homozygous null fibroblasts, it was demonstrated that TGFβ-mediated transcriptional activation of 3TP-Lux (35) and the cJun promoter (14), which contain both Smad and AP-1 DNA-binding sites, was lost in fibroblasts lacking Smad3. Transfection of Smad3 into the null fibroblasts rescued transcriptional activation by TGFβ. Here, we further show that activation of the AP-1 sites in a reporter containing mutant Smad-binding sites similarly requires Smad3. Taken together, the interaction and transcriptional activation data suggest that the regulated association between Smads and AP-1 may be necessary for the TGFβ-mediated transcriptional activation of AP-1 sites. Consistent with these findings, recently published data demonstrate that cotransfection of Smad3 and Smad4 and cJun facilitates mild synergistic transcriptional activation of AP-1-site containing reporters (36).

We demonstrate that Smad3 and Smad4 bind JunB directly *in vitro,* and that the interaction involves a stretch of 20 C-terminal amino acids of JunB. It is possible that deletion of these 20 amino acids that abrogated Smad binding may alter the conformation of JunB, rendering it incapable of binding Smads. However, we do show that both cJun and JunD, which contain conserved identity in 13 of these 20 C-terminal amino acids, also bind GST-Smad3 and GST-Smad4, whereas Fos family members that lack this conserved amino acid sequence do not bind GST-Smad3 or GST-Smad4. Therefore, we postulate that Smad3 and Smad4 binding requires specific residues within the C-terminal 20 amino acids of JunB. Furthermore, direct association with Smads may be a conserved function of the Jun family of transcription factors.

Because Smads translocate to the nucleus in response to TGFβ (20–23), the interaction between Smads and Jun family members may be regulated largely by TGFβ-induced alteration of Smad subcellular localization. Intriguingly, we found that endogenous cJun was rapidly phosphorylated in response to TGFβ, most likely at Ser-63. Phosphorylation at this site peaks by 15 min and is maintained over the course of an hour of TGFβ treatment, a time course that overlaps TGFβ-induced Smad entry into the nucleus (17, 21, 22, 25). This inducibly phosphorylated form of cJun binds both GST-Smad3 and GST-Smad4. Although this phosphorylation does not appear to alter the association of Smads and AP-1 *in vitro*, we suspect this phosphorylation may contribute to TGFβ-mediated transcription *in vivo.* This notion is supported by evidence showing that JNK is activated in response to TGFβ (37, 38), and that this kinase is known to phosphorylate cJun at Ser-63 and -73, thereby enhancing the ability of cJun to activate transcription (39). These data, in combination with previously discussed results, suggest that TGFβ treatment may initiate two simultaneous signaling pathways that converge on AP-1 complexes in the nucleus: a Smad-mediated pathway and a JNK-mediated pathway. The combined result of these pathways may be stronger interactions between Smads and AP-1 and as a result, a more robust induction of transcription. Although cJun phosphorylation may indeed enhance association with Smads under physiological conditions, we do not have evidence to suggest that Jun phosphorylation is required for the Smad/AP-1 interaction, since we observe the interaction under *in vitro* conditions in which JNK-mediated phosphorylation would not occur and also detect a constitutive interaction of GST-Smads with endogenous cJun in the absence of TGFβ treatment. Furthermore, JunB, which is not a JNK substrate, also binds to Smads (40). Future work is necessary to define the role of Jun phosphorylation in TGFβ-mediated transcription *in vivo.*

The data presented here may provide a plausible explanation for the specificity of TGFβ-mediated induction of specific responsive promoters that contain AP-1 DNA-binding sites. On TGFβ treatment, Smad3 and Smad4 heteromerize and enter the nucleus, where they can associate with TGFβ-responsive promoters by binding a discreet DNA sequence and/or AP-1 members bound to AP-1 sites on the same promoter. Thus, Smads in response to TGFβ act as the signaling intermediates to initiate transcription from specific promoters by recruiting required factors to form an active transcriptional complex. The transcriptional adapter molecule p300/CBP, which binds directly to AP-1 and serves as a coactivator of AP-1-mediated transcription, has recently been shown to associate directly with Smads in response to TGFβ (41–44). TGFβ-induced Jun modification may promote the stability of these interactions, thereby facilitating complex formation. Given that AP-1 complex composition depends on differential expression of specific family members, the distinct constitution of AP-1 complexes in different cell types may contribute to promoter targeting specificity by TGFβ. This possibility is supported by the observation that under conditions where Smads bind Jun proteins, Smads are unable to associate with Fos family members. Thus, it is possible that AP-1 complexes containing Fos members may have a lower affinity for Smads than AP-1 complexes containing Jun–Jun dimers, and that promoters associated with these specific AP-1 complexes would be favored for Smad binding and TGFβ-mediated activation. The induced interaction of Smads with particular AP-1 complexes *in vivo* may determine the ability of

TGFβ to initiate transcription from specific AP-1 site-containing promoters.

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TGF-β–induced Phosphorylation of Smad3 Regulates Its Interaction with Coactivator p300/CREB-binding Protein

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Smads are intermediate effector proteins that transduce the TGF-β signal from the plasma membrane to the nucleus, where they participate in transactivation of downstream target genes. We have shown previously that coactivators p300/CREB-binding protein are involved in TGF-β–mediated transactivation of two Cdk inhibitor genes, p21 and p15. Here we examined the possibility that Smads function to regulate transcription by directly interacting with p300/CREB-binding protein. We show that Smad3 can interact with a C-terminal fragment of p300 in a temporal and phosphorylation-dependent manner. TGF-β–mediated phosphorylation of Smad3 potentiates the association between Smad3 and p300, likely because of an induced conformational change that removes the autoinhibitory interaction between the N- and C-terminal domains of Smad3. Consistent with a role for p300 in the transcription regulation of multiple genes, overexpression of a Smad3 C-terminal fragment causes a general squelching effect on multiple TGF-β–responsive reporter constructs. The adenoviral oncoprotein E1A can partially block Smad-dependent transcriptional activation by directly competing for binding to p300. Taken together, these findings define a new role for phosphorylation of Smad3: in addition to facilitating complex formation with Smad4 and promoting nuclear translocation, the phosphorylation-induced conformational change of Smad3 modulates its interaction with coactivators, leading to transcriptional regulation.

INTRODUCTION

TGF-β is a growth factor that regulates various cellular functions in many cell types (Lyons and Moses, 1990; Massague, 1990; Roberts and Sporn, 1993). Central to this is its ability to inhibit cellular proliferation by causing an arrest in the G1 phase of the cell cycle. In addition, TGF-β regulates the expression of many cellular genes involved in extracellular matrix production and turnover. Clues to the molecular mechanisms through which TGF-β exerts these cellular effects have come from the discovery of the Smad family of proteins.

Smads are intermediate effector molecules of the signaling pathways of the TGF-β superfamily of ligands. To date, at least nine Smads have been cloned (Heldin *et al.*, 1997; Hu *et al.*, 1998; Massague, 1998). Among them, the highly related Smad2 and Smad3 are specific effectors for TGF-β signaling (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996), and Smad 4 is a common partner for TGF-β superfamily signaling (Hahn *et al.*, 1996; Lagna *et al.*, 1996). Smad 2 and most likely Smad3 are phosphorylated at their extreme C terminus (SSVS) by type I receptor during TGF-β treatment (Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996). This phosphorylation overcomes the autoinhibitory state of Smad2 between its N and C terminus, promoting its interaction with Smad4 and subsequent translocation to the nucleus (Hata *et al.*, 1997). In addition, overex-

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pression of Smad3 and Smad4, which presumably leads to higher absolute levels of basally phosphorylated forms of these proteins, can cause ligand-independent transcriptional activation of certain TGF-β–inducible genes such as plasminogen activator inhibitor 1 (PAI-1) (Zhang *et al.*, 1996); however, the mechanism leading to transcriptional activation is still largely unknown. Smads have been shown to bind DNA directly (Kim *et al.*, 1997; Yingling *et al.*, 1997), and this ability to bind to DNA may correlate, at least in part, with transcriptional activity inherent to Smad molecules and/or in conjunction with coactivator partners (Liu *et al.*, 1997; Dennler *et al.*, 1998; Zawel *et al.*, 1998).

Clues to the biological functions of Smads have also come from the discovery that certain Smads are tumor suppressors mutated in human cancers. Smad4 was originally identified as a tumor suppressor on chromosome 18q, termed DPC4, which is mutated in 50% of human pancreatic cancers (Hahn *et al.*, 1996). Smad4 mutations and deletions have been discovered in other types of cancers, including breast, ovary, head, and neck, and esophageal cancers (Barrett *et al.*, 1996; Kim *et al.*, 1996; Nagatake *et al.*, 1996; Schutte *et al.*, 1996). Smad2 is also defined as a tumor suppressor gene because its mutations have been found in colon and head and neck cancers (Eppert *et al.*, 1996). These findings suggest a role for Smads in cell growth regulation and have lead to the hypothesis that the Smads may be central regulators of TGF-β–mediated growth inhibition (Massague, 1998).

Regulation of the cell cycle in the G1 phase is dependent on the activity of cyclin-dependent kinase (Cdk) complexes, primarily the cyclin D-Cdk4/Cdk6 and cyclin E-Cdk2 complexes. TGF-β has been shown to cause cell cycle arrest by inhibiting the Cdk activities in certain cell types by inducing the expression of the two Cdk inhibitors p15 and p21 (Hannon and Beach, 1994; Datto *et al.*, 1995a; Reynisdottir *et al.*, 1995). To probe the signaling mechanism by which TGF-β regulates cell cycle progression, we previously mapped the TGF-β–responsive elements of the p15 and p21 promoters to Sp1 binding sites in HaCaT cells (Datto *et al.*, 1995a,b; Li *et al.*, 1995). Subsequently, we found that canonical Sp1 binding sites can function as a distinct TGF-β–responsive element for TGF-β–mediated promoter expression, and Sp1 protein, but not family member Sp3, can mediate this response (Li *et al.*, 1998a).

In a separate study, we demonstrated that the coactivator p300 is required for the TGF-β–mediated induction of p15 and p21 (Datto *et al.*, 1997). p300 is a phosphoprotein that was first discovered in anti-E1A cellular immunoprecipitates (Eckner *et al.*, 1994), and it has a functional homologue, CREB-binding protein (CBP), that also binds to E1A (Chrivia *et al.*, 1993). In HaCaT cells, the ability of E1A to abolish TGF-β–mediated growth inhibition, in addition to its binding and inactivation of the retinoblastoma protein Rb, appears to stem from its binding to p300/CBP, which prevents TGF-β–mediated induction of p15 and p21 and relieves cyclin–Cdk repression (Missero *et al.*, 1995; Datto *et al.*, 1997). Although p300/CBP was shown to be required for p15 and p21 induction, the mechanism by which its activity is modulated by the TGF-β signal remains unresolved.

Because p300/CBP appears to be essential in TGF-β–mediated growth inhibitory signaling and because the Smads, by their nature as tumor suppressors, have also been implicated in growth control, we chose to explore the possibility of a functional or physical interaction between these proteins. In this report, we show that Smad3 interacts with p300 in a temporal and TGF-β–regulated phosphorylation-dependent manner. Thus, Smad3 may play a role as a mediator of the TGF-β growth inhibitory signaling pathway. This notion is supported by the recent finding that overexpression of Smad3 and Smad4 could lead to a dramatic ligand-independent transactivation of the p21 promoter in a hepatic cell line (Moustakas and Kardassis, 1998). Furthermore, we provide evidence that the interaction between Smad3 and p300 may be essential for the transcriptional responses of multiple target genes to TGF-β. Specifically, the Smad-dependent induction of the PAI-1 gene by TGF-β is blocked by E1A but not by an E1A mutant deficient in p300 binding, implicating the interaction between Smad3 and p300 as an important requirement for TGF-β signaling.

MATERIALS AND METHODS

Antibodies and Reagents

Human TGF-β1 was a generous gift from Amgen. Anti-HA was from Boehringer Mannheim (Indianapolis, IN). Anti-Smad3 antibody was generated against a specific peptide (DAGSPNLSPNPMSPAHNNLD) in the linker region of Smad3 and purified in this laboratory; anti-Smad4 (sc-7966) and anti-p300 (sc-584 AC) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). TNT SP6-coupled reticulocyte lysate system was from Promega (Madison, WI). Calf intestine alkaline phosphatase (CIAP) and potato acid phosphatase (PAP) were from Boehringer Mannheim.

Cell Culture

Human HaCaT cells were a generous gift from Drs. P. Baukamp and N. Fusenig (Institute of Biochemistry and Molecular Biology, Heidelberg, Germany). They were grown in MEM supplemented with 10% FBS and 2 mM L-glutamine (Life Technologies, Gaithersburg, MD). COS cells were maintained in DMEM with 10% FBS.

Plasmids

HA-tagged Smad3 has been described previously (Yingling *et al.*, 1996). pCMV5-Smad3 C-HA (aa 199–424), Smad4C-HA (aa 266–552), Smad3-Flag, Smad3NL-Flag, Smad3CΔC-Flag, and Smad3ΔC-Flag were generous gifts from Dr. Rik Derynck (Zhang *et al.*, 1997). GST-p300M (aa 744-1571) and GST-p300C (aa 1572–2414) were gen-

erous gifts from Dr. Yang Shi (Lee *et al.*, 1995). PAI-1-Luc (Zhang *et al.*, 1996), 3TP-Lux (Wrana *et al.*, 1992), p15P113-Luc (Li *et al.*, 1995), p21P-Luc (Datto *et al.*, 1995b), and Gl1xkB (Li *et al.*, 1998b) have been described previously.

GST Pull-down Assays

The bacterial strain TOPP1 containing GST-p300M and GST-p300C was grown in 5 ml of Luria broth media overnight at 37°C. The next day, the cultures were transferred to flasks containing 50 ml of Luria broth and shaken vigorously for 1 h (optical density, ~0.6) at 37°C. Isopropylthio-β-D-galactaside (0.5 mM) was then added to the culture and shaken vigorously for another 3 h at 37°C. Cells were sonicated four times on ice in 30 s intervals. Lysates were clarified by centrifugation at 7000 rpm before addition of 200 μl of a 50% slurry of lysis buffer-equilibrated glutathione beads. After a 4 h incubation at 4°C, the beads were pelleted by centrifugation at 1000 rpm and washed three times in lysis buffer before resuspension in 1 ml of lysis buffer. In GST pull-down assays, equal amounts of cell lysates or in vitro translated product were incubated with immobilized GST beads in lysis buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethyl-sulfonyl fluoride and protease inhibitors) at 4°C for 2 h. After the beads were washed four times with lysis buffer, the bound proteins were eluted by boiling in 1× Laemmli sample buffer and subjected to immunoblot analysis. For phosphatase treatment, cell lysates were made with or without phosphatase inhibitor 1 mM sodium orthovanadate, 50 mM NaF, 20 mM β-glycerophosphate, and 0.1 mM sodium molybdate, and then treated with 2 U CIAP and 2 μg PAP at 37°C for 15 min. Thirty micrograms of treated lysates were used as control in immunoblotting, and the rest of the lysates were used for GST pull-down assays.

Luciferase Assays

Transfections were performed by using a standard DEAE–dextran transfection protocol (Li *et al.*, 1995). Briefly, 150,000 cells were plated onto each well of a six-well plate and grown overnight. The cells were then washed once with PBS and incubated in serum-free MEM containing 100 μM chloroquine. The DEAE–dextran mixture containing DNA was then added to the cells and incubated for 3 h. The cells were then glycerol-shocked for 2 min and incubated in medium containing 10% FBS. Twelve hours after transfection, 100 pM TGF-β1 was added, and TGF-β–induced luciferase activity was assayed after 24 h. Luciferase assays were performed as described previously (Li *et al.*, 1995).

Immunoprecipitation and Western Blot Analysis

Cells after treatment were harvested in lysis buffer described above. Agarose-conjugated p300 antibodies (5 μl) were added into ~300 μg of lysate and incubated at 4°C for at least 3 h. The beads were washed three times with 0.5 ml of lysis buffer. Then loading buffer containing *N*-ethylmaleimide instead of DTT was added and incubated at room temperature for 20 min to shift the heavy chain of antibodies to a higher position before loading on the gel for Western blot analysis.

Proteins from HaCaT lysates or transfected COS lysates were resolved by SDS-PAGE and transferred to Immobilon-P (Millipore, Bedford, MA). The membranes were then blocked in 5% nonfat milk in 1× PBS and 0.1% Tween 20. The blots were incubated with primary antibody in block solution for 1 h at room temperature and subsequently washed three times in PBS/Tween. The appropriate secondary antibody was added for 1 h at room temperature. After three washes with PBS/Tween, the immunoreactive proteins were visualized by ECL (Amersham, Buckinghamshire, UK) and autoradiography.

RESULTS

Smad3 Binds the p300 C-Terminal Fragment

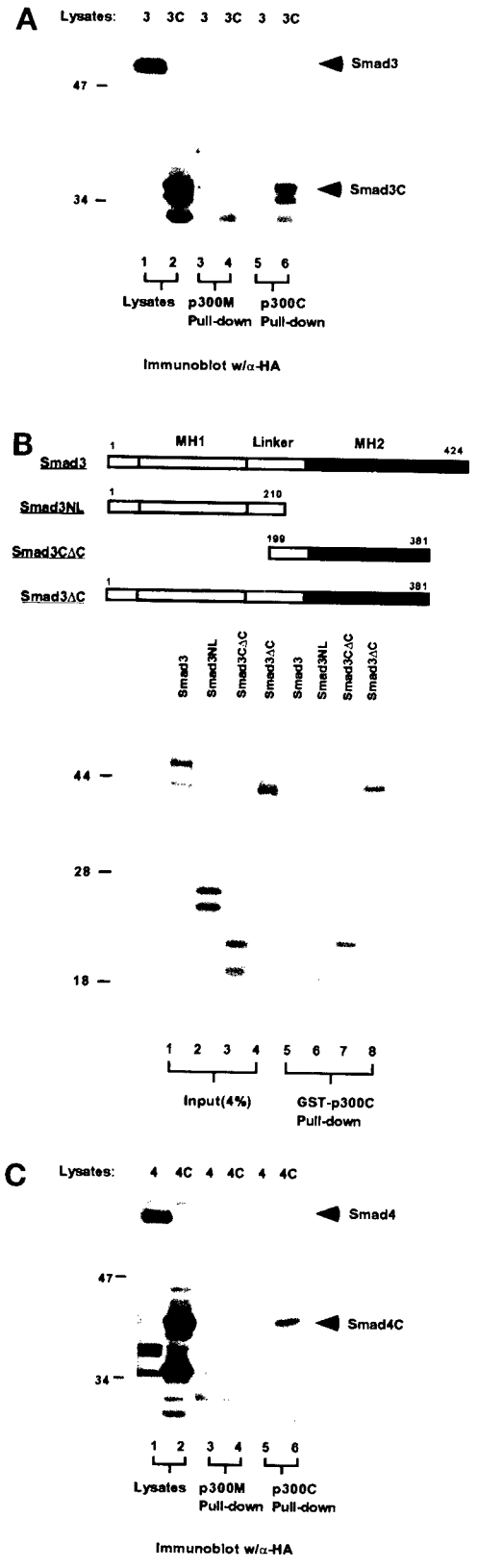
To examine whether p300 and Smad3 can interact with each other, we performed pull-down experiments using two p300 fragments, GST-p300M (aa 744-1571) and GST-p300C (1572–2414) (Lee *et al.*, 1995), with COS cell lysates containing overexpressed HA-tagged Smad3 and the N-terminal–truncated Smad3 (aa 199–424), termed Smad3C. As shown in Figure 1A, Smad3C can interact strongly with GST-p300C but not GST-p300M. Intriguingly, the full-length Smad3 interacts only weakly with both GST-p300M and GST-p300C.

To further define the region of interaction on Smad3, as well as to determine whether this association is direct, ^{35}S-labeled in vitro-translated full-length Smad3 and the indicated fragments were used to perform additional pull-down experiments with GST-p300C (Figure 1B). Consistent with the pull-down experiment with COS lysates, full-length Smad3 and the Smad3 N-terminal fragment were found to interact weakly with p300C, in comparison with the strong interactions between p300C and Smad3ΔC or p300 and Smad3CΔC. This suggests that the region of interaction with p300 is between aa 199 and 381 of Smad3. Most importantly, these results indicate that deletion of either the N or distal C terminus of Smad3 can strongly enhance its interaction with p300, suggesting that the unmodulated conformation of full-length Smad3 may be inaccessible to p300 interaction.

To determine whether p300 could also interact with Smad4, the binding partner of Smad3, we repeated the GST-p300C pull-down experiments with COS lysates containing overexpressed Smad4 (aa 1–552) and Smad4C (aa 266–552). As shown in Figure 1C, p300 was found to associate with Smad4C but not with full-length Smad4. This result suggests that Smad4C, when overexpressed, also has the ability to interact with p300.

TGF-β Induces the Association between Smad3 and p300

Because either the N- or the C-terminal truncated Smad3 protein fragment interacts with p300 more strongly than full-length protein, we reasoned that the conformation of unstimulated Smad3 is likely to be autoinhibitory in a manner similar to that previously demonstrated for Smad2 (Hata *et al.*, 1997). Hence, TGF-β type I receptor-mediated phosphorylation of the SSVS motif in the C-terminal region of Smad3 and subsequent relief of the autoinhibited conformation of this protein are necessary for the interaction with p300 to occur. To test this hypothesis, we treated HaCaT cells with TGF-β for increasing lengths of time and used GST-p300C to pull down endogenous Smad3. The total amount of Smad3 protein did not change



with up to 4 h of TGF-β treatment of these cells (Figure 2A). At time 0, we found that GST-p300C did not interact with endogenous Smad3; however, in lysates from cells treated with TGF-β for 10 min up to 2 h, GST-p300 was readily able to interact with endogenous Smad3. Complex formation between Smad3 and p300 peaks at 30 min and completely diminishes by the 4 h time point (Figure 2A). This time course of observed interaction parallels that for Smad phosphorylation after TGF-β treatment (Yingling *et al.*, 1996). This result strongly suggests that the interaction between Smad3 and the coactivator p300 is a TGF-β–regulated event that correlates directly with the phosphorylation of Smad3.

To further probe the mechanism underlying the TGF-β–induced temporal association between Smad3 and p300, we treated HaCaT lysates with phosphatases (CIAP and PAP) to determine whether phosphorylation was the underlying event required for this association. In the phosphatase-treated lysates of cells incubated with TGF-β for 30 min, the interaction of Smad3 with GST-p300C was almost completely abolished (Figure 2B). It is also of note that without exogenous phosphatase treatment, this interaction is greatly reduced in the absence of phosphatase inhibitors in the lysis buffer (Figure 2B, lane 7) and suggests that this reduced association is a result of Smad3 dephosphorylation by endogenous phosphatases. As a control, it is demonstrated that the total amount of Smad3 protein is not affected by the indicated phosphatase incubation conditions. These results demonstrate that the TGF-β–induced conformational change of Smad3, most likely through the phosphorylation of Smad3 at its C-terminal region, is required for its interaction with p300. This notion is further supported by the results shown in Figure 1B, in which it is demonstrated that Smad3ΔC and Smad3CΔC, both of which lack the SSVS site of phosphorylation, have a much stronger affinity for p300C, and suggests that these sites of phosphorylation are not required for the interaction of Smad3 with p300 but rather that the

Figure 1. p300 C-terminal region interacts with Smad3C and Smad4C. (A) p300C interacts with Smad3C. HA-tagged full-length Smad3 and Smad3 C-terminal fragment (aa 199–424) constructs were transfected into COS cells as indicated. Cells were harvested 48 h after transfection, and GST pull-downs were performed using GST-p300M (aa 744-1571) and GST-p300C (aa 1572–2414). The bound proteins were analyzed by immunoblotting with antibodies against HA. The lysates in lanes 1 and 2 represent ~12% of the amount used for the pull-down. (B) A region between aa 199 and 381 of Smad3 interacts with p300. Smad3 and truncated forms were in vitro-translated using rabbit reticulocyte lysates. Ten microliters of the ^{35}S-labeled proteins were incubated with GST-p300C beads for 2 h at 4°C, and the bound proteins were analyzed by SDS-PAGE followed by fluorography. (C) Smad4C can interact with p300C. HA-Smad4 and Smad4C (aa 266–552) were transfected into COS cells, and the lysates were pulled down with GST-p300C as in A.

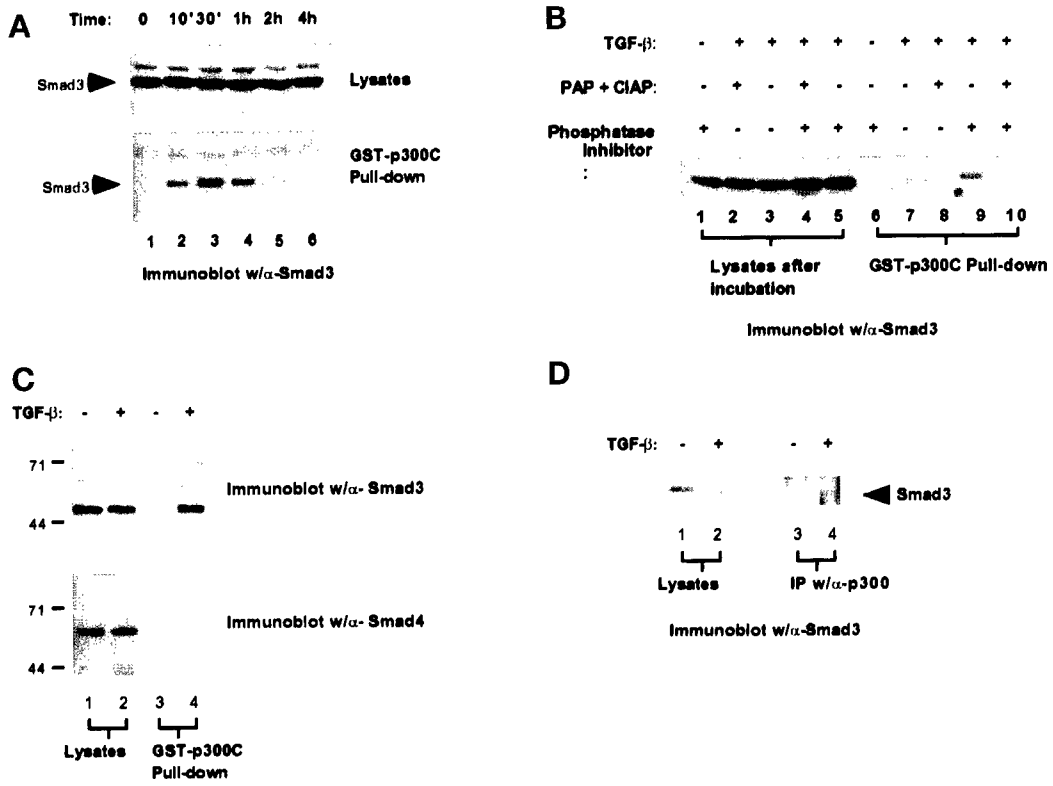


Figure 2. TGF-β regulates the interaction of Smad3 and p300 in a temporal and phosphorylation-dependent manner. (A) The time course of interaction between Smad3 and p300 after TGF-β treatment. HaCaT cells were treated with TGF-β for 0–4 h. Total cell lysates were used for the GST-p300C pull-down assay as described in MATERIALS AND METHODS. Bound proteins were separated by SDS-PAGE and immunoblotted with antibodies against Smad3. (B) The association of Smad3 and p300 is phosphorylation dependent. HaCaT cells were treated with TGF-β for 0 and 30 min, and then lysates were treated with phosphatases CIAP and PAP, as described in MATERIALS AND METHODS, and used for the GST-p300C pull-down assay as in A. Bound proteins were separated by SDS-PAGE and immunoblotted with antibodies against Smad3. (C) Endogenous Smad3, but not Smad4, interacts with GST-p300C after TGF-β treatment. HaCaT lysates treated with TGF-β for 0 and 30 min were precipitated by GST-p300C and then immunoblotted with antibodies against Smad3 and Smad4. (D) Smad3 interacts with p300 in vivo. HaCaT cells were incubated with TGF-β for 30 min. Lysate (300 μg) was used for immunoprecipitation using agarose-conjugated antibodies against p300 and then immunoblotted with antibodies against Smad3. Thirty micrograms of lysates were loaded on the gel to shown the correct size of Smad3.

phosphorylation-induced conformational change of Smad3 is the essential event.

We also determined whether Smad4, when expressed at endogenous levels, can bind to p300 in a TGF-β–regulated manner in the same system. In HaCaT lysates either untreated or incubated with TGF-β for 30 min, Smad4 was not able to associate with GST-p300C, whereas Smad3 was TGF-β–inducibly associated with p300 in the same experiment (Figure 2C). Thus, although both Smad3 and Smad4 have the potential to interact with p300 as demonstrated by the COS overexpression experiment (Figure 1, A and C), only endogenous Smad3 but not Smad4 can interact with p300 during TGF-β treatment. This is probably because only Smad3 can undergo phosphorylation during TGF-β treatment, which will lead to a conformational change favorable for the interaction with p300.

To demonstrate an in vivo interaction between Smad3 and p300, we performed immunoprecipitation and Western blot analysis using HaCaT cell lysates. Cells untreated or treated with TGF-β for 30 min were harvested, immunoprecipitated with agarose-conjugated p300 antibodies, and blotted with the anti-Smad3 antibody. As shown in Figure 2D, the association between Smad3 and p300 is observed only after TGF-β treatment, a result fully consistent with that of the GST pull-down assay. Taken together, these results indicate that Smad3 interacts with p300 in a temporal and ligand-induced phosphorylation-dependent manner.

Overexpression of Smad3C Has a Squelching Effect on Multiple TGF-β–regulated Promoters

To further explore the functional significance of the interaction between Smad3 and p300, we tested whether the overexpression of Smad3C (aa 199–424), a Smad3 fragment that can constitutively bind to p300, could affect TGF-β–mediated transactivation of multi-

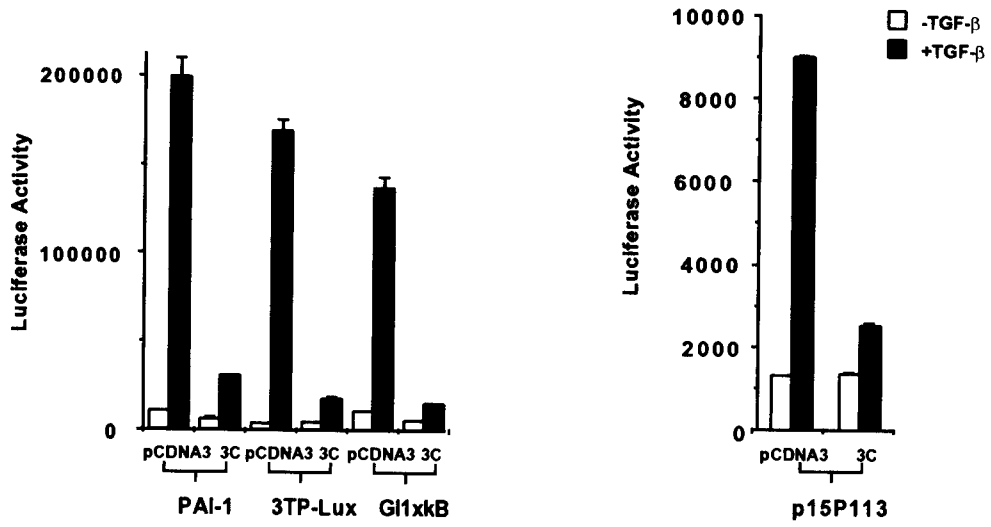


Figure 3. Overexpression of Smad3C has a squelching effect on multiple TGF-β–responsive reporter genes. Three micrograms of HA-tagged Smad3C were cotransfected with 3 μg of different reporter constructs into HaCaT cells as indicated. The total DNA amount was kept constant by adding pCDNA3.

ple target genes. As shown in Figure 3, cotransfection of Smad3C with PAI-1-Luc, 3TP-Lux, GI1XκB, a minimal responsive reporter construct containing NFκB sites (Li *et al.*, 1998a,b), and the minimal promoter for the p15 gene, p15P113-Luc, caused a dramatic decrease in TGF-β–induced transcriptional activity. This broad spectrum of transcriptional inhibition by the overexpressed Smad3C may be the result of a sequestration of a common factor, likely the coactivator p300/CBP, although we cannot rule out the possibility that titration of endogenous Smad4 or other factors also plays a role in this process. These results nevertheless suggest that p300, and possibly the interaction between Smad3 and p300, may be required for the mediation of TGF-β–signaling pathways leading to the activation of multiple genes involving different families of transcription factors.

E1A Competes with Smad3 for Binding to p300

We and others have shown previously that the adenoviral oncoprotein E1A is able to antagonize TGF-β–mediated transcription and growth inhibition (Pietenpol *et al.*, 1990; Missero *et al.*, 1991; Abraham *et al.*, 1992; Datto *et al.*, 1997). This activity of E1A is dependent on its ability to bind to two main target proteins, p300/CBP and pRB. The demonstration here that Smad3 interacts with p300 in a temporal and phosphorylation-dependent manner indicates that this interaction may be important for the transactivation ability of Smads. To test whether E1A can act to block Smad-mediated transcription activation in a p300-dependent manner, we examined the effect of E1A on the TGF-β–induced expression of PAI-1-Luc and 3TP-Lux, two reporters that have been shown to require Smads for transcriptional activation. As shown in Figure 4A, E1A can dramatically inhibit the TGF-β–mediated transactivation of these two promoters in HaCaT cells cotransfected with either of these two reporter constructs. Furthermore, the inhibitory effect of E1A on the TGF-β induction of the two promoters was significantly reduced when HaCaT cells were cotransfected with an E1A mutant, Δ2–36, that is severely attenuated in p300 binding (Kraus *et al.*, 1992; Wang *et al.*, 1993). Both promoters have been previously shown to be transcriptionally activated in a ligand-independent manner during cotransfection of Smad3 and Smad4. Consistent with the notion that Smad3 and Smad4 play a role as effectors for TGF-β in this transactivation event by binding to p300, E1A greatly reduced the 20-fold ligand-independent transactivation of the PAI-1 reporter resulting from cotransfected Smad3 and Smad4, whereas cotransfection of the mutant E1A, Δ2–36, only partially affected the Smad3/Smad4 ligand-independent effect (Figure 4B).

Because the E1A binding site of p300 has been previously mapped to the C-terminal region, which is now shown to interact with Smad3, we next tested whether E1A acts to affect TGF-β–induced transcription by competing with Smad3 for p300 binding. Consistent with this model, increasing amounts of bacterially produced 6XHis-tagged E1A decreased the ability of Smad3C to interact with GST-p300C in an in vitro binding assay (Figure 4C). This result implicates a mechanism by which E1A antagonizes TGF-β–mediated transcriptional activation and growth inhibition through its competition with Smad3 for binding to the coactivator p300.

DISCUSSION

In this report, we present data supporting a model for the mechanism by which Smads function to activate

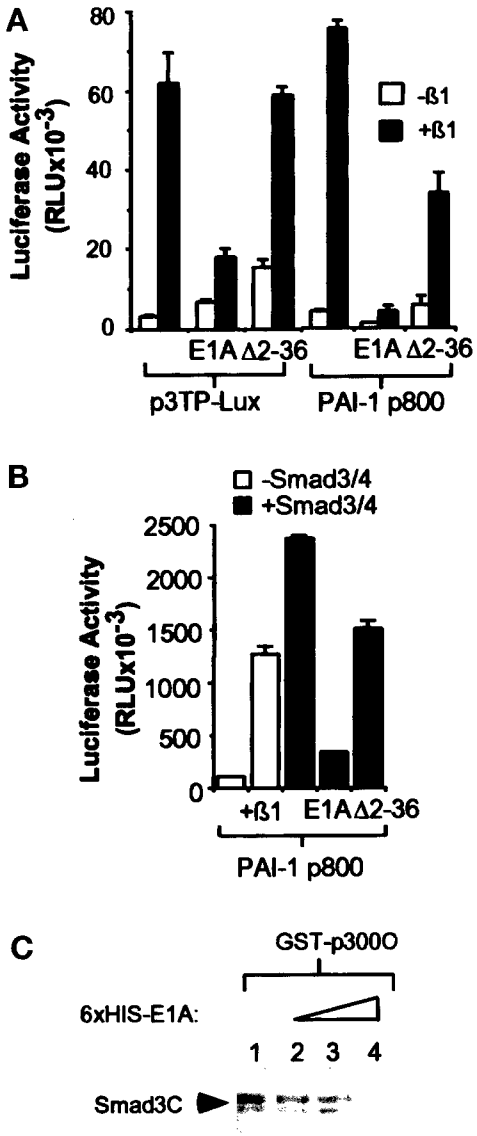


Figure 4. E1A inhibits Smad-dependent transcriptional activation. (A) E1A, but not the p300 binding mutant E1AΔ2–36, blocks transcriptional activation of 3TP-Lux and PAI-1-Luc. HaCaT cells were cotransfected with 3 μg of 3TP-Lux or PAI-1-Luc reporter constructs and 4 μg of the indicated E1A expression constructs. The total amount of DNA was kept constant with the addition of vector control pCDNA3. TGF-β was added 12 h after transfection, and luciferase activity was measured 24 h later. Error bars represent the SD for duplicate transfections in a single experiment. "E1A" stands for wild-type E1A, and "Δ2–36" stands for E1AΔ2–36 mutant. (B) The transcriptional activation induced by Smad3/Smad4 overexpression is inhibited by E1A in a p300-dependent manner. HaCaT cells were cotransfected with 3 μg of PAI-1, 3 μg of Smad3/Smad4, and 4 μg of E1A expression constructs as indicated. The total DNA amount was kept constant with the addition of pCDNA3. After transfection and TGF-β treatment, luciferase activity was measured as above. (C) E1A can compete with Smad3 for interaction with p300. COS-overexpressed HA-tagged Smad3C (aa 199–424) was used to access the ability of Smad3 to interact with p300 in the presence of E1A. Eluted bacterial-produced 6XHis E1A was added in increasing amounts from lanes 2 to 4 to the GST-p300C pull-down reaction. After incubation at 4°C for 2 h, the bound proteins were washed three times with lysis buffer and immunoblotted with antibodies against HA.

transcription through a TGF-β–regulated interaction with coactivator p300/CBP. In this model (Figure 5), TGF-β treatment initiates a kinase cascade that results in the phosphorylation of Smad3, followed by its heteromerization with Smad4 and subsequent translocation into the nucleus. Once in the nucleus, phosphorylated Smad3 can interact with the coactivator p300/CBP, and likely other transcription factors, to activate transcription from TGF-β target genes. In this sequence of signaling events, the differential association of Smad3 with p300/CBP in a temporal and phosphorylation-dependent manner plays a key role in the regulatory mechanism by which TGF-β activates the transcription of downstream genes. In this context, E1A can prevent the Smad3-dependent activation of target promoters by competing with Smad3 for p300/CBP binding. This model is supported by three recent reports demonstrating the interaction between Smad2 or Smad3 and p300/CBP (Feng *et al.*, 1998; Janknecht *et al.*, 1998; Topper *et al.*, 1998).

Transcriptional activation in general can be regulated at multiple levels: de novo synthesis of a transcription factor, translocation of the transcription factor from cytosol to nucleus, or posttranslational modification. In the case of Smad-mediated signaling, both changes in localization and phosphorylation play a role in their ability to transactivate downstream genes during TGF-β treatment. One model suggests that phosphorylation of the three C-terminal serine residues on Smad2 (SSVS) by the TGF-β type I receptor changes Smad2 conformation to a state in which the Smad2 N-terminal arm, which normally acts to inhibit its biologically active C terminus, dissociates from the C terminus. This, in turn, promotes the association of phosphorylated Smad2 with Smad4 and subsequent translocation of the complex into the nucleus (Hata *et al.*, 1997). Building on this working model, our results suggest that aside from its role in complex formation and nuclear translocation, phosphorylation-induced conformational change is also important for Smad3 nuclear function in terms of promoting interaction with p300/CBP. This may be explained by the possibility that the p300-binding domain of Smad3 is masked when it is in an unphosphorylated autoinhibited conformation. As for Smad4, we were unable to show that endogenous Smad4 interacts with p300/CBP during TGF-β treatment, probably because of the lack of phosphorylation during the treatment; however, because Smad4 is known to interact with Smad3 in a DNA binding complex during TGF-β treatment, it is very likely that Smad4 is contained in a functional complex containing

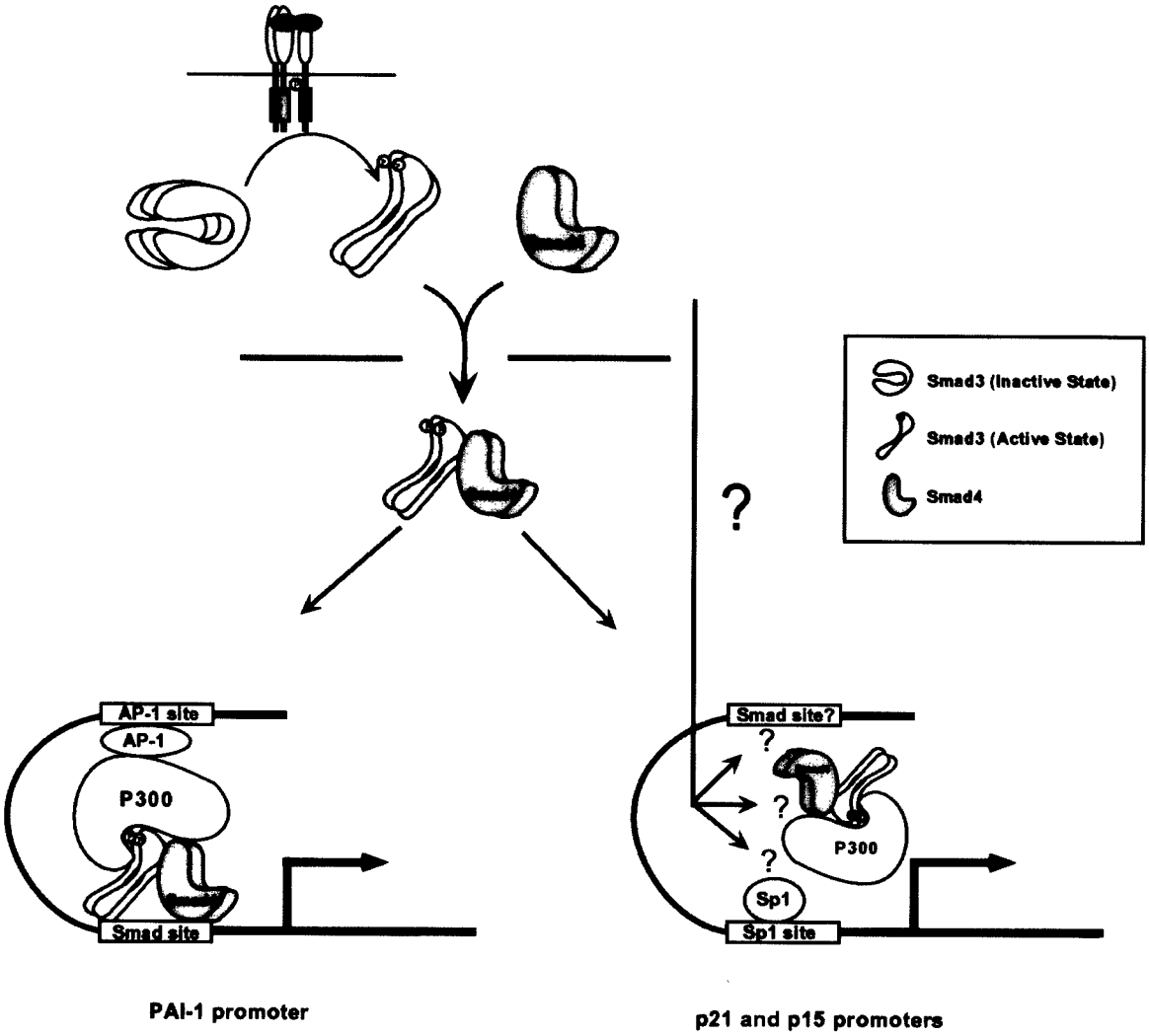


Figure 5. Proposed model for Smad-dependent TGF-β signal transduction pathway. TGF-β treatment initiates a receptor kinase cascade that results in the phosphorylation of Smad3. The phosphorylation of Smad3 weakens the interaction between the N and C terminal regions of Smad3, enabling its interaction with Smad4 and subsequent nuclear translocation of the complex. Once in the nucleus, Smad3 is able to associate with p300 because of the phosphorylation-induced unmasking of the p300 interaction region of Smad3. The Smad3-p300/CBP interaction synergizes with AP1-p300/CBP interaction to activate transcription for PAI-1 promoter. The mechanism underlying the TGF-β–induced transactivation of the p21 and p15 promoters may require both the Smad3-p300/CBP interaction and additional signals that may modulate the interaction between Sp1 and the rest of the transcriptional complex.

both p300/CBP and Smad3. The detection of Smad4 in such a complex may be difficult in our pull-down experiments because the interaction is through Smad3. Furthermore, an excess amount of GST-p300C could potentially interfere with the association between Smad3 and Smad4.

Once in the nucleus, Smads may cooperate with the coactivator p300/CBP, as well as other transcription factors, to recruit the basal transcriptional machinery to the promoter to initiate transcription. Smads may direct the formation of such higher order complexes to specific promoters through their direct binding to specific DNA sequences (Chen *et al.*, 1997; Yingling *et al.*, 1997; Dennler *et al.*, 1998), as well as potentially to other transcription factors, such as Fos and Jun of the AP-1 complex, a possibility implicated by our previous work suggesting a necessary functional interaction between Smads and AP-1 in the transactivation of the 3TP-Lux reporter (Yingling *et al.*, 1997). Indeed, both the p3TP-Lux and PAI-1 promoters contain Smad-specific binding sequences as well as AP-1 elements that appear to be important in modulating the TGF-β and Smad-dependent responses (Yingling *et al.*, 1997; Dennler *et al.*, 1998). It is also worth noting that both Fos and Jun can directly associate with p300/CBP (Arias *et al.*, 1994) and consequently strengthen

the interactions among different components in the preinitiation complex. After it is recruited to specific promoters, p300/CBP may also help to stabilize the preinitiation complex by making additional contacts with TBP and TFIIB (Kwok *et al.*, 1994; Swope *et al.*, 1996; Dallas *et al.*, 1997). Recent studies have suggested an important enzymatic function for p300/CBP as a histone and protein acetyltransferase, paramount to its ability to initiate transcription (Ogryzko *et al.*, 1996; Gu and Roeder, 1997). In this model, binding of p300/CBP to transcription factors, such as Smad3/Smad4 and Jun/Fos, may allow its acetyltransferase activity to acetylate surrounding histones, thereby loosening the chromatin and increasing the accessibility of the preinitiation complex to DNA.

Many other transcription factors also require p300 and CBP for transcriptional activation (Arias *et al.*, 1994; Bhattacharya *et al.*, 1996; Chakravarti *et al.*, 1996; Kamei *et al.*, 1996). Because cellular concentrations of p300 and CBP are limited, one would expect that these transcription factors will compete for p300 and CBP. This has been demonstrated in steroid hormone signaling where overexpression of the nuclear receptor for steroid hormone can inhibit phorbol-ester–activated transcription from AP-1 sites by competing for p300 and CBP (Kamei *et al.*, 1996). Consistent with this, as well as with the finding that a specific region of Smad3 can interact strongly with p300/CBP, transcriptional activation of multiple TGF-β–responsive promoters was dramatically inhibited during Smad3C overexpression (Figure 3), suggesting that p300/CBP may play a critical role in TGF-β signaling. In contrast to the constitutively active Smad2C reported in a previous study (Baker and Harland, 1996), overexpressed Smad3C is inhibitory in our experimental system, possibly because of its sequestration of p300/CBP. This discrepancy could reflect the different molecular characteristics of Smad2 and Smad3 as reported recently: the opposite effect of Smad2 and Smad3 on the transcription of mouse *goosecoid* gene through binding to FAST2 (Labbe *et al.*, 1998). In addition, different expression levels of these two proteins in the two assaying conditions could also lead to a different outcome in those functional assays. In our transient transfection experiment, for example, an inhibitory effect is observed only when >0.5 μg of Smad3C is transfected; below this amount of transfected DNA, transcription on 3TP-Lux reporter actually increases slightly (our unpublished results).

Functional disruption of Smads and p300/CBP is thought to contribute to the loss of cell cycle control and carcinogenesis (Muraoka *et al.*, 1995; Borrow *et al.*, 1996; Eppert *et al.*, 1996; Hahn *et al.*, 1996). In this regard, the exact role of Smads in the mediation of the growth inhibitory effect of TGF-β, and their connection to transcriptional activation of p15 and p21, two important effectors in TGF-β–mediated growth arrest, are just beginning to be understood. The squelching effect of Smad3C on the transcriptional activation of p15 minimal promoter in response to TGF-β suggests that Smad3 may be required for TGF-β–induced expression of the p15 gene, and consequently TGF-β–mediated cell cycle arrest. To test this possibility, we cotransfected Smad2, Smad3, and Smad4 in various combinations to determine whether overexpression of Smads can activate transcription of the two Cdk inhibitor genes, in comparison to that of the positive control, the PAI-1 promoter. Our results indicate that overexpression of Smad3 and Smad4, or other combinations of different Smads, could not potentiate transcription from the p15 and p21 promoters, whereas the PAI-1 promoter is greatly activated by Smad3 and Smad4 overexpression (our unpublished results). This result is in contrast to the recent report that Smad3 and Smad4 coexpression could potently activate the p21 promoter in a hepatic cancer line, HepG2 (Moustakas and Kardassis, 1998). The discrepancy between the two apparently opposite results is most likely due to the difference in the cell types used in the studies. It is conceivable that a putative, essential signal that acts in conjunction with overexpressed Smads to initiate transcription of the p21 promoter is constitutively active in the HepG2 cells, and in contrast, is only TGF-β–inducible in the HaCaT cells used in this study. This hypothesis is consistent with the observation reported by Moustakas *et al.* that expression of endogenous p21, as well as activation of the p21 promoter luciferase construct, is constitutively high in HepG2 cells, whereas in HaCaT cells, endogenous p21 levels are barely detectable in untreated culture yet markedly induced by TGF-β. Therefore, although the function of Smads as intermediates of TGF-β signaling may be essential for multiple pathways, the mode of their involvement in transcriptional activation of specific target genes may mechanistically differ in various cell types. In addition, within a distinct cell type such as HaCaT cells, specific TGF-β–responsive genes may require different stimuli for p300-dependent transcription to occur. For example, in HaCaT cells, Smad overexpression alone is sufficient to stimulate transcription of the PAI-1 promoter, yet not that of p21 or p15 genes. For these promoters, Smad overexpression and subsequent nuclear translocation is only one essential component of the complete TGF-β signal. Other distinct, yet to be defined signaling events that are apparently constitutively active in HepG2 cells, yet only TGF-β inducibly so in HaCaT cells, are also required to cooperate with Smads/p300/CBP to fully activate transcription from these promoters.

Combined with other studies, our results suggest a general strategy by which signal-dependent transcriptional activation can occur for a once seemingly disparate group of transcription factors that include Smads, Stats, and NF-κB (Darnell, 1997; Zhong *et al.*,

1998). During stimulation by specific external signals, these transcription factors are phosphorylated and change conformation, form complexes with partner proteins or dissociate from inhibitory sequestration, and translocate from the cytosol into the nucleus. Once in the nucleus, they bind to the coactivator p300 or CBP in a phosphorylation-dependent manner to activate transcription. This general transcriptional activation strategy may be an evolutionarily conserved mechanism that transduces extracellular stimuli into a prompt transcriptional response.

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Targeted Disruption of Smad3 Reveals an Essential Role in Transforming Growth Factor β-Mediated Signal Transduction

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The Smads are a family of nine related proteins which function as signaling intermediates for the transforming growth factor β (TGF-β) superfamily of ligands. To discern the in vivo functions of one of these Smads, Smad3, we generated mice harboring a targeted disruption of this gene. Smad3 null mice, although smaller than wild-type littermates, are viable, survive to adulthood, and exhibit an early phenotype of forelimb malformation. To study the cellular functions of Smad3, we generated Smad3 null mouse embryonic fibroblasts (MEFs) and dermal fibroblasts. We demonstrate that null MEFs have lost the ability to form Smad-containing DNA binding complexes and are unable to induce transcription from the TGF-β-responsive promoter construct, p3TP-lux. Using the primary dermal fibroblasts, we also demonstrate that Smad3 is integral for induction of endogenous plasminogen activator inhibitor 1. We subsequently demonstrate that Smad3 null MEFs are partially resistant to TGF-β's antiproliferative effect, thus firmly establishing a role for Smad3 in TGF-β-mediated growth inhibition. We next examined cells in which Smad3 is most highly expressed, specifically cells of immune origin. Although no specific developmental defect was detected in the immune system of the Smad3 null mice, a functional defect was observed in the ability of TGF-β to inhibit the proliferation of splenocytes activated by specific stimuli. In addition, primary splenocytes display defects in TGF-β-mediated repression of cytokine production. These data, taken together, establish a role for Smad3 in mediating the antiproliferative effects of TGF-β and implicate Smad3 as a potential effector for TGF-β in modulating immune system function.

Transforming growth factor β (TGF-β) is a multifunctional polypeptide hormone which has diverse effects on a variety of cell types to regulate many complex multicellular systems (46). The complexity and diversity of TGF-β's function is demonstrated through its multiple roles in immune system suppression, wound healing, fibrosis, development, and oncogenesis. Many of these global effects of TGF-β stem from its ability to regulate cellular proliferation, differentiation, and gene expression (38). One of the most studied aspects of TGF-β function is its ability to inhibit the proliferation of many different cell types, including cells of epithelial, endothelial, neuronal, hematopoietic, and lymphoid origins (46, 31).

These effects of TGF-β are mediated through its interaction with cell surface receptors. By binding to its type I and type II serine/threonine kinase receptors, TGF-β induces the phosphorylation and activation of the type I receptor by the type II receptor (57, 58). The type I receptor kinase can then phosphorylate cytoplasmic substrates, including members of the Smad family of proteins, which function as intermediates in the signaling pathways for the TGF-β superfamily of ligands (3, 4, 12, 18, 39). Originally identified in genetic screens for TGF-β effectors in *Drosophila* (49) and *Caenorhabditis elegans* (47), the mammalian Smad family now consists of nine structurally related proteins, Smad1 to Smad9. The identification and characterization of these proteins has provided valuable insights into the early events involved in TGF-β-mediated signal transduction.

The highly related Smad2 and Smad3 serve as substrates for the type I TGF-β receptor kinase (14, 30, 36, 42, 53, 62, 65). Upon phosphorylation, these two Smads bind to their common partner, Smad4, to form Smad2-Smad4 and Smad3-Smad4 complexes. These complexes then translocate to the nucleus (1, 29, 33, 41, 66). Clues to the nuclear function of these Smad complexes came from studies describing an intrinsic transcriptional activity of the C-terminal domain of the Smads (29, 59). Subsequently, overexpression of particular combinations of Smads was shown to activate transcription from a number of TGF-β-responsive promoters, including the plasminogen activator inhibitor 1 (PAI-1) promoter and the reporter construct 3TP-lux (11, 29, 65).

The role of the Smads as putative transcription factors was strengthened by the finding that Smad3-Smad4 complexes and the *Drosophila* Mad are sequence-specific DNA binding proteins which on binding DNA can activate transcription (11, 25, 63, 64). In addition to a direct DNA binding activity, the Smads can be targeted to specific promoter sequences through their interaction with other transcription factors, as demonstrated by the finding that Smad2-Smad4 complexes bind to the transcription factor FAST-1 in response to activin and TGF-β (7, 34). In addition, recent studies have implicated a functional interaction between the Smad3-Smad4 complex and the AP1 family of transcription factors (32, 63, 67).

Apace with the rapid development of the understanding of the Smads on a biochemical level, the role of the Smads in development and diseases is beginning to be understood. Recently, mouse models for both Smad2 and Smad4 function

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have been described (43, 52, 55, 61). Mice with homozygous targeted disruptions of these genes are embryonic lethal at day 9.5 and days 6.5 to 8.5, respectively. Thus, these Smads play critical, nonredundant roles in early embryonic development. The early embryonic lethality of these mice, however, renders the functional analysis of these molecules in the adult animals impossible in this system and makes their study on a cellular level difficult.

In humans, the role of Smad2 and Smad4 as tumor suppressor genes is now well established (6, 14–16, 40, 44, 45, 48). Concurrent with the identification of the Smads through genetic screens, Smad4 was identified as a tumor suppressor gene, which is deleted in about 50% of pancreatic carcinomas. In addition to pancreatic cancers, Smad4 mutations have also been discovered in breast, ovary, head and neck, esophagus, colon, and lung cancers. Not only are Smad4 mutations found in spontaneous cancers, but recent reports show that inherited juvenile colon cancer can derive from the inheritance of a single mutant Smad4 allele (19). In addition, Smad2 is mutated in several types of cancers, including colon cancers and head and neck cancers (14, 44). To date, Smad3 has not been reported to be mutated in human cancers (2, 45). These data, together with their role as intermediates in the TGF-β signaling pathway, clearly implicate Smad2 and Smad4 as playing an important function in cell growth regulation.

The cellular functions of the Smads have largely been inferred from the occurrence of mutations in human diseases and from cellular studies employing the use of Smad dominant negatives and Smad overexpression in Smad-deficient cell lines which likely harbor additional genetic lesions. Thus, the physiological functions of Smad3, particularly its potential involvement in mediating the TGF-β antiproliferative effect, remain speculative. To address the biological functions of Smad3, we generated mice harboring a targeted disruption of the Smad3 gene. Unlike the Smad2 and Smad4 null mice, Smad3 null mice are viable and survive to adulthood, demonstrating distinct roles for the three Smad proteins during mouse development. In addition, Smad3 null mice are smaller than wild-type littermates and have an incompletely penetrant joint formation abnormality. At the cellular level, we focused our study initially on defining the role of Smad3 in TGF-β signal transduction in the mouse embryonic fibroblast (MEF) and dermal fibroblast model systems. Here we show that Smad3 is required for activation of a TGF-β-responsive promoter, 3TP-lux, and the endogenous PAI-1 gene and, more importantly, acts as an integral effector of TGF-β-mediated inhibition of cellular proliferation. We next focused on the cell types with highest Smad3 expression, specifically cells of lymphoid origin, and found that under specific conditions, the antiproliferative effects of TGF-β on isolated Smad3 null splenocytes are lost. In addition, we found that the inhibition of anti-CD3 (αCD3)-stimulated cytokine production by TGF-β in primary splenocytes is markedly blunted due to the absence of Smad3. Taken together, these findings implicate Smad3 as a critical effector in TGF-β-mediated inhibition of cellular proliferation and a potential effector for TGF-β regulation of immune system function.

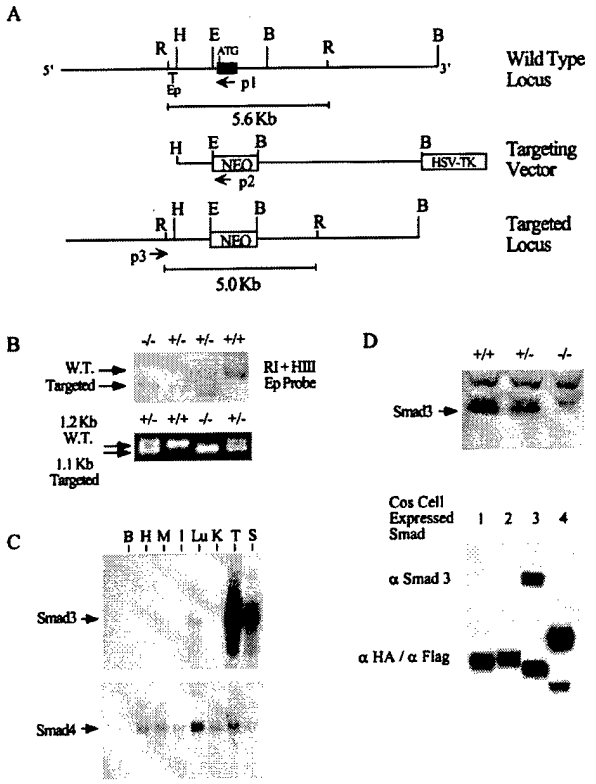

FIG. 1. Targeted disruption of Smad3. (A) Smad3 genomic structure and targeting strategy. The Smad3 genomic clone used for the creation of the targeting vector is diagrammed. The black box denotes the first exon of Smad3. ATG denotes the initiating methionine. The first exon coding sequence was replaced by a neomycin expression cassette (NEO), creating an *Eco*RI digest size difference between the wild-type and targeted loci. Ep denotes the *Eco*RI-*Hin*dIII DNA fragment used as a probe for Southern blotting. P1, P2, and P3 denote the locations of primers used for PCR screening. Restriction sites are abbreviated as follows: R, *Eco*RI; H, *Hin*dIII; E, *Ehe*I; B, *Bam*HI. HSV-TK, herpes simplex virus thymidine kinase. (B) Southern and PCR detection of the targeted allele. The targeted allele can be distinguished from the wild type (W.T.) both by Southern blotting of *Eco*RI-digested genomic DNA with the Ep probe and by PCR using the primers indicated in panel A. (C) Northern blot analysis of Smad3 and Smad4. Northern analyses were performed on RNA derived from multiple tissues of an adult (2-month-old) C57BL/6 mouse and a 3′ untranslated sequence probe for Smad3 and a coding-sequence probe for Smad4. Organs are abbreviated as follows: B, brain; H, heart; M, skeletal muscle; I, small intestine; Lu, lung; K, kidney; T, thymus; S, spleen. (D) Western blot analysis of Smad3 expression. The top panel shows Western analysis using an antibody created against a peptide in the central linker domain of Smad3 on thymic protein extract from wild-type, heterozygous, and knockout mice. The bottom two panels demonstrate the specificity of this antibody among overexpressed Smad family members. HA-tagged Smad1, Smad2, and Smad4 and Flag-tagged Smad3 were overexpressed in COS cells from which protein extract were isolated and used for Western analysis. Identical blots were probed with the Smad3-specific antibody (middle panel) and a mixture of αHA and αFlag (bottom panel). In all panels of all figures, +/+ denotes Smad3 wild type, +/− denotes Smad3 heterozygous, and −/− denotes Smad3 null.

MATERIALS AND METHODS

Smad3 gene disruption. The Smad3 gene was isolated from a 129/sv mouse genomic library by using the 5′ end of the human Smad3 cDNA as a probe. An isolated 15-kb genomic clone was used for the creation of a Smad3 targeting vector. Briefly, a 1.0-kb *Ehe*I-*Hin*dIII fragment was cloned into the *Xho*I site of the vector pPNT (54). A 6.0-kb *Bam*HI fragment was next cloned into the resulting construct. This produced a targeting vector which, when inserted into the genome, replaces the sequence between *Ehe*I and *Bam*HI with a neomycin expression cassette, as diagrammed in Fig. 1A. The resulting targeting vector was linearized with *Hin*dIII and electroporated into 129/sv embryonic stem (ES) cells. Screening of neomycin-resistant clones was performed by PCR with the following primers: the common primer (P3; GTC TTT GAG GCC CGT TTT CTG C), a primer from the targeted sequence (P1; CTG GGG TGG TAA TGC ACT TGG), and a primer in the *PGK* promoter (P2; CAT GCT CCA GAC TGC CTT GGG). PCR of the wild-type allele results in a 1.2-kb product. PCR of the targeted allele results in a 1.1-kb product. Positive clones were confirmed by Southern blotting of *Eco*RI-digested genomic DNA probed with an *Eco*RI-*Hin*dIII fragment immediately adjacent to the sequences used in the targeting vector. The wild-type allele of Smad3 produces a 5.6-kb fragment. The targeted allele produces a 5.0-kb fragment.

Primary fibroblast and immune cell culture. Primary fibroblasts were cultured from day 14 embryos. Embryos were mechanically disrupted by passage through an 18-gauge needle and plated on gelatin-coated 10-cm-diameter plates in Dulbecco modified Eagle medium (DMEM) with 20% heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin (P-S) (Gibco BRL, Gaithersburg, Md.). Confluent cells were trypsinized and further carried in medium containing 10% FBS. In all experiments, compared wild-type and null cells represent littermate embryos at the same passage number.

Primary dermal fibroblasts were isolated from 2-day-old mice. Trunk skin was removed, washed three times in phosphate-buffered saline (PBS) containing kanamycin, amphotericin, penicillin, and streptomycin (KAPS), and incubated overnight at 4°C in 0.25% trypsin (Worthington Biochemical, Freehold, N.J.) in PBS-KAPS. The skins were then incubated at 37°C for 20 min. The trypsin was next neutralized with 20% FBS, and the skins were then washed in DMEM–10% FBS. The trypsinized skins were next placed in individual 10-cm-diameter dishes, and the epidermis was peeled off and discarded. The resulting dermis layers were mechanically dissociated, and 10 ml of DMEM–10% FBS–P-S was added to each dish. Cells were then incubated at 37°C in 5% CO_2 until dermal fibroblasts became confluent (3 to 4 days). The dermal fibroblasts were carried in DMEM–10% FBS–P-S and genotyped, and passage 5 cells were used in the PAI-1 assay.

Primary splenocytes, thymocytes, and purified splenic B and T cells were generated from the spleens of 2- to 4-month-old mice. Lymphocytes were isolated by mechanical dissociation in the culture medium described below. Large debris was removed, and erythrocytes were lysed by complete resuspension of pelleted cells in 145 mM ammonium chloride–17 mM Tris (pH 7.5). Cells were subsequently washed in PBS and resuspended in their final culture medium for all experiments described: RPMI with the addition of 10% heat-inactivated FBS, P-S, and 0.1 mM β-mercaptoethanol.

Northern and Western blotting. Northern blotting for Smad3 was performed on RNA prepared from adult C57BL/6 mouse organs by homogenizing the indicated tissues in Trizol reagent (Gibco BRL, Gaithersburg, Md.) as specified by the manufacturer. Ten micrograms of total RNA was resolved on a formaldehyde-agarose gel, which was subsequently transferred by capillary action to a nylon membrane (Hybond, Amersham Life Science) and visualized by methylene blue staining to confirm RNA loading and quality. Blots were probed with a mouse Smad3 cDNA probe created by random priming (Prime-It II, Stratagene, La Jolla, Calif.) of a *Hin*dIII-*Eco*RI fragment containing sequences entirely in the Smad3 3′ untranslated region. The membrane was subsequently reprobed with a human Smad4 cDNA probe containing the entire coding sequence for human Smad4.

For Western blotting, all cell and organ lysates were prepared in a Nonidet P-40-based lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 50 mM NaF, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 0.2 mM sodium molybdate, protease inhibitors). For Western blotting of thymus tissue, whole thymus was homogenized in 1 ml of lysis buffer and equal protein amounts from animals of each genotype were analyzed. Western blotting of fibroblasts was performed by lysis of 10^6 fibroblasts from 10-cm-diameter tissue culture plates in 200 μl of lysis buffer and analyzing equal protein amounts for each genotype. Western analyses of equal amounts of protein extracts from splenic B cells purified by using Dynabeads- Mouse pan T (Dynal, Lake Success, N.Y.) and of splenic T cells purified by using mouse T-cell enrichment columns (R&D Systems, Minneapolis, Minn.) were performed with a Smad3-specific antibody. Western analyses of αCD3-stimulated splenocytes were performed on splenocytes isolated as described above and cultured at a density of 10^7 cells in 2 ml of medium in six-well tissue culture plates in the presence 5 μg of αCD3 (01081D; PharMingen, San Diego, Calif.) with and without 100 pM TGF-β for 24 (cyclin E cyclin-dependent kinase [Cdk2], and p27 western blots) or 48 h (retinoblastoma protein [Rb] Western blots). Cells were lysed in 200 μl of lysis buffer, and equal protein amounts were analyzed. Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western analyses were performed with the following antibodies; αcyclin E (M-2), αCdk2 (M-2), αp27 (C-19), αp21 (C-19), αp15 (C-20), and αCDC25A (144), all from Santa Cruz Biotechnology, Inc., and αRb (14001A; PharMingen). Smad3 Western analyses were performed with a rabbit polyclonal antibody created against the Smad3-specific peptide DAGSPNLSPNPMSPAHNNLD. Crude rabbit serum was further purified on an antigen affinity column. The specificity of this antibody was assessed by Western analysis of equal protein amounts of COS cell extract in which the following tagged human Smads were individually overexpressed: Flag-tagged Smad1, Flag-tagged Smad2, hemagglutin epitope (HA)-tagged Smad3, and Flag-tagged Smad4. Cytomegalovirus promoter-driven constructs encoding the various Smads were transiently transfected into COS cells by using a standard DEAE-dextran protocol as previously described (10). Expression of the various Smads was confirmed by Western analysis of equal amounts of appropriately transfected COS extract, using a mixture of αHA (Boehringer Mannheim, Indianapolis, Ind.) and αFlag (Eastman Kodak, New Haven, Conn.) antibodies.

Cdk2 kinase assays. Splenocytes were isolated and cultured as described above for Western blotting. Whole-cell lysates were generated after 24 h of TGF-β treatment in the same manner as for Western analysis. Cdk2 kinase activity immunoprecipitated with a polyclonal αCdk2 antibody (M-2; Santa Cruz Biotechnology) from equal protein amounts for each condition described was measured on the substrate histone H1 as previously described (10).

PAI-1 assay. Dermal fibroblasts were plated at a density of 10^6 cells per 10-cm-diameter plate in the fibroblast culture medium described above and incubated overnight at 37°C. Cells were then incubated in methionine-free DMEM–0.5% FBS–P-S for 4 h and then treated with 100 pM TGF-β for 6 h. During the last 2 h of TGF-β treatment, the fibroblasts were labeled with [^{35}S]methionine (100 μCi/ml). The cells were then washed with PBS and removed by lysis in three washes of 10 mM Tris-HCl (pH 8.0)–0.5% sodium deoxycholate–1 mM phenylmethylsulfonyl fluoride, and the resulting plate-bound extracellular matrix washed a final time with PBS. The amount of matrix associated PAI-1 was assessed by scraping the plates in SDS-PAGE loading buffer containing dithiothreitol and resolving the protein on an 10% polyacrylamide gel. Gels were subsequently dried, and autoradiography was performed.

Thymidine incorporation assays. Fibroblasts of the indicated genotypes were plated at a density of 20,000 cells/well in six-well tissue culture plates in DMEM–10% FBS and incubated in the presence or absence of 100 pM TGF-β for 24 or 48 h as indicated. For the last 4 h of culture, 5 μCi of [^{3}H]thymidine was added to the culture, and thymidine incorporation was assayed as previously described (10). For mixed wild-type and Smad3 null experiments, the indicated percentage of each cell type was plated in six-well plates to a total cell number of 20,000/ml. Thymidine incorporation was assayed after 48 h as described above.

Thymidine incorporation of splenocytes was performed on cells isolated as described above. Isolated splenocytes were plated at a density of 5×10^5 cells in 200 μl of medium in 24-well plates and stimulated with lipopolysaccharide (LPS; 10 μg/ml; Sigma, St. Louis, Mo.), anti-immunoglobulin M (αIgM; 5 μg/ml; Cappel, Durham, N.C.) and interleukin-4 (IL-4; 12.5 U/well; PharMingen) or αCD3 (2.5 μg/ml; PharMingen) in the presence or absence of 100 pM TGF-β and cultured for 48 h; 5 μCi of [^{3}H]thymidine was added to the culture for the last 4 h. Thymidine incorporation was assayed by harvesting cells with a PHD cell harvester (Cambridge Technologies, Inc.).

Luciferase assays. Fibroblasts (200,000/well) from each genotype were seeded into six-well tissue culture plates. Cells were transfected by using a standard DEAE-dextran transfection protocol as previously described (10) with the indicated amounts of DNAs (Fig. 4B). Cells were cotransfected with 0.25 μg of a cytomegalovirus-driven β-galactosidase reporter vector to normalize for transfection efficiency. Transfections with the expression and reporter plasmids used here have been previously described (63).

EMSAs. For electrophoretic mobility shift assays (EMSAs), nuclear extracts prepared from 10^6 fibroblasts of the indicated genotype either treated or untreated with 100 pM TGF-β for 1 h were incubated with a probe derived from an *Nde*I-*Sph*1 fragment of 3TP-lux (63). Nuclear extract preparation and gel shift conditions were exactly as previously described (62).

RNase protection analysis of cytokine expression. Primary splenocytes were isolated as described above. Splenocytes (7.5×10^6) were plated in 2 ml of medium and stimulated with 5 μg of αCD3 in the presence or absence of 100 pM TGF-β for 48 h in individual wells of a six-well plate. The cells were harvested, and total RNA was isolated (RNeasy; Qiagen, Santa Clarita, Calif.). Cytokine RNA levels were assessed by RNase protection assays using a RiboQuant multiprobe kit (45024K/mCK-1; PharMingen) on equal amounts of RNA (7.5 μg) for each culture condition as specified by the manufacturer. Equal amounts and quality of RNA were confirmed through the quantification of the protection fragments of two housekeeping genes provided in the multiprobe template set, L32 and *GAPDH*.

FACS analysis. Fluorescence-activated cell sorting (FACS) analysis was performed on live splenocytes and thymocytes isolated as described above. Approximately 10^5 pelleted cells were resuspended in 100 μl of PBS with 5% heat-inactivated FBS with the inclusion of the antibodies indicated in each figure: phycoerythrin (PE)-αCD4 (01065B), fluorescein isothiocyanate (FITC)-αCD8 (01044D), and FITC-αB220 (01124D) (all from PharMingen), plus PE-αIgM and 7-amino actinomycin D (7AAD; Molecular Probes, Eugene, Oreg.). After 30 min, these cells were washed once in PBS–5% FBS and analyzed. The purity of the isolated splenic B and T cells used in the thymidine incorporation assays was assessed by FACS analysis with the use of PE-αB220 and FITC-labeled anti-T-cell receptor beta chain (01304D and 01125B, respectively; PharMingen). Viable cells were identified by exclusion of 7AAD (Molecular Probes) staining.

RESULTS

Smad3 null mice are viable. To generate a targeted disruption of Smad3, we first screened a murine 129/sv genomic library with sequences in the amino terminus of Smad3 to obtain a 14-kb genomic clone. This clone contains the first exon of Smad3, including the initiating methionine and the first 69 amino acids. A targeting vector was created by replacing the first exon and part of the first intron with a *PGK*-neomycin expression cassette. Proper insertion of this targeting vector into the mouse genome removes the initiating ATG, making the production of full-length Smad3 impossible. In addition, this insertion does not disrupt any sequences 5′ to the RNA transcriptional start site (Fig. 1A).

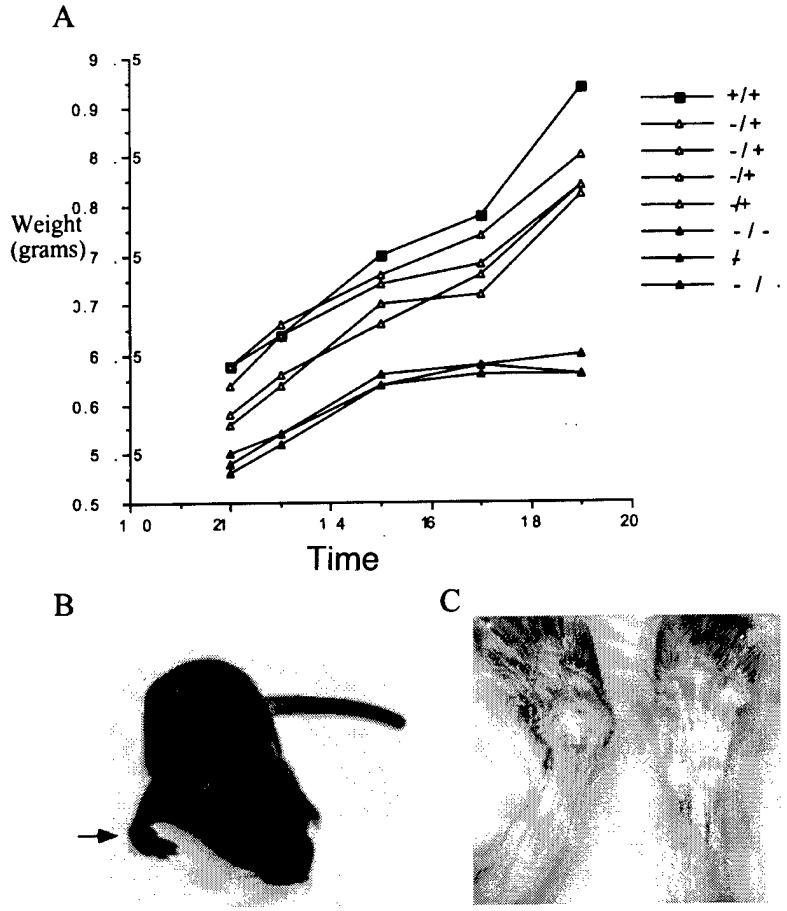

FIG. 2. Smad3 null mice are smaller than wild-type littermates and have an incompletely penetrant forepaw defect. (A) Mouse weights over time (days) in a single, representative litter. (B) Picture of the torqued-wrist defect (arrow) in a 14-day-old null mouse. (C) The skin was removed from the forelimbs of a 14-day-old Smad3 null mouse (left) and a wild-type littermate (right) to better show the severe bending of the forepaw wrist joint of the Smad3 null mouse.

Using standard ES cell technology, Smad3 mutant heterozygous 129 ES lines were generated by transfection of the described targeting vector. Initial screening for proper insertion in neomycin-resistant clones was determined by Southern blotting and PCR as indicated in Fig. 1B. Three percent of neomycin-resistant ES cell clones had a properly targeted Smad3 allele. These ES cells were then used to create 129-C57BL/6 chimeric founder mice. When bred to C57BL/6 females, mice generated from one of these lines transmitted the mutant Smad3 allele at a frequency of 50%, with 100% of offspring being derived from the 129 stem cells. Heterozygous mice from these matings were subsequently mated to produce Smad3 null mice. Smad3 null mice are born to F_1 heterozygotes at a frequency of 20.7%, the same frequency as for wild-type mice (297 Heterozygote, 106 wild-type, and 103 KO knockout mice). The near Mendelian inheritance of wild-type and targeted Smad3 alleles suggests no embryonic lethality of the Smad3 null mice. Thus, in sharp contrast to the Smad2 and Smad4 deficiencies, Smad3 is not essential for embryonic development. This F_2 generation of mice was used for the experiments described below.

To identify organs with highest Smad3 expression, we first performed multiple-tissue Northern analysis. Unlike Smad2 and Smad4, Smad3 has an expression pattern which varies with tissue types, with highest levels of expression in the spleen and thymus (Fig. 1C). Subsequently, the loss of Smad3 expression in the double-mutant animals was confirmed by Western analysis of thymus protein extracts, using a Smad3-specific antibody (Fig. 1D).

The first noticeable phenotype in these null animals is a decrease in the size and growth rate of young mice. As shown in Fig. 2A, Smad3 null mice are smaller than both wild-type and heterozygous littermates. An additional early phenotype, which occurs in approximately 31% (32 of 103) of null mice, is the presence of medially torqued forepaws (Fig. 2B and C), with a smaller percentage of mice with noticeably torqued hind limbs. Mice with this phenotype can have either one or more limbs affected. In addition, mice with severely affected limbs often develop kyphosis and display marked rib cage malformation often resulting in a concave indentation at the base of the sternum (data not shown). Interestingly, this phenotype is remarkably similar to that of mice expressing a transgenic dominant negative type II TGF-β receptor in bone (50), suggesting that the phenotype described here is intrinsic to the bone. In addition, the similarity between these two phenotypes suggests that the previously described TGF-β effects in bone development are at least partially mediated by Smad3.

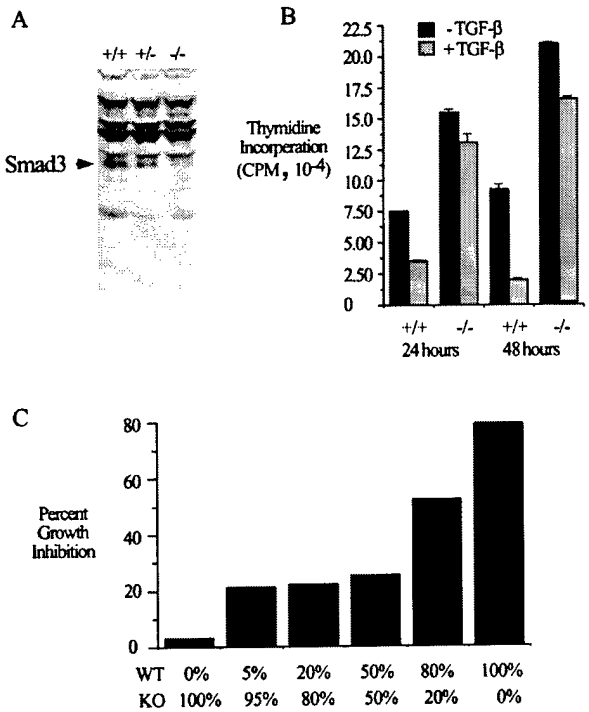

FIG. 3. Smad3 is required for TGF-β-mediated growth inhibition in MEFs. (A) Primary MEFs were created from embryonic day 14 mice. Western blotting for Smad3 was performed to determine if these MEFs express Smad3. (B) Smad3 is required for TGF-β-mediated growth inhibition in primary MEFs. MEFs were assayed for TGF-β-mediated growth inhibition after 24 and 48 h of treatment by measurement of [^{3}H]thymidine incorporation. Bars represent the average thymidine incorporation for triplicate wells for each growth condition. (C) TGF-β-mediated growth inhibition in these MEFs is cell autonomous. Various proportions of wild-type (WT) and knockout (KO) MEFs were seeded into single wells as indicated below the bars. Thymidine incorporation assays were performed as for panel B. Data are presented as percent growth inhibition or percent reduction in thymidine incorporation upon TGF-β treatment.

TGF-β-mediated growth inhibition and gene responses are impaired in Smad3 null fibroblasts. One functional aspect of Smad3 that we hoped to define through the generation of Smad3 null mice is its role in TGF-β-mediated inhibition of cellular proliferation. To test this, we isolated MEFs from both wild-type and Smad3 null mice. As shown in Fig. 3A, Smad3 expression can be detected in wild-type fibroblast lines but not in lines derived from Smad3 null embryos. Using these fibroblasts, we first determined the proliferative responses of these lines to TGF-β. As shown in Fig. 3B, the proliferation of wild-type fibroblasts is inhibited approximately 50% after 24 h and 80% after 48 h of TGF-β treatment. In null fibroblasts, this growth-inhibitory effect of TGF-β is largely lost. In addition, the basal proliferation rate of the null fibroblast lines is approximately twofold higher than that of the wild type. Similar results were obtained for two additional fibroblast lines of each genotype (data not shown). Thus, these results firmly establish an essential role for Smad3 in TGF-β-mediated inhibition of cellular proliferation. Interestingly, none of the known mediators of the growth-inhibitory effect of TGF-β appear to be functioning in fibroblasts. In these cells TGF-β does not alter p21, p15, or CDC25A protein levels, whereas p27 is undetectable (data not shown).

To determine if the growth-inhibitory effect of TGF-β in these cultures is cell autonomous or due to inappropriately regulated production of paracrine factors, growth inhibition by TGF-β of mixed wild-type and null cultures was assayed. As shown in Fig. 3C, different percentages of wild-type and null cells were seeded into the same well, and TGF-β mediated growth inhibition was assayed. The growth-inhibitory effect of TGF-β in these experiments is proportional to the amount of wild-type cells. This suggests that the antiproliferative effect of TGF-β in these cells is most likely cell autonomous and not due to a Smad3-dependent production of growth-inhibitory or inhibition of growth-stimulatory paracrine factors.

As discussed above, the Smads have been characterized as DNA binding transcription factors. To determine the requirement of Smad3 in the activation of specific promoters, we studied the regulation of the widely used TGF-β-responsive promoter 3TP-lux in our model fibroblast system. In wild-type cells, the previously described TGF-β-induced, Smad3-containing DNA binding complex forms on the concatemerized tetradecanoyl phorbol acetate response elements (TREs) present in this promoter. This DNA binding complex is lost in the Smad3 null fibroblasts (Fig. 4A). In addition, transcription from this promoter in wild-type cells is activated 2.4-fold upon TGF-β treatment. This activation is lost in the null fibroblasts and can be restored by cotransfection of a Smad3 expression vector (Fig. 4B). Thus, Smad3 is necessary not only for the

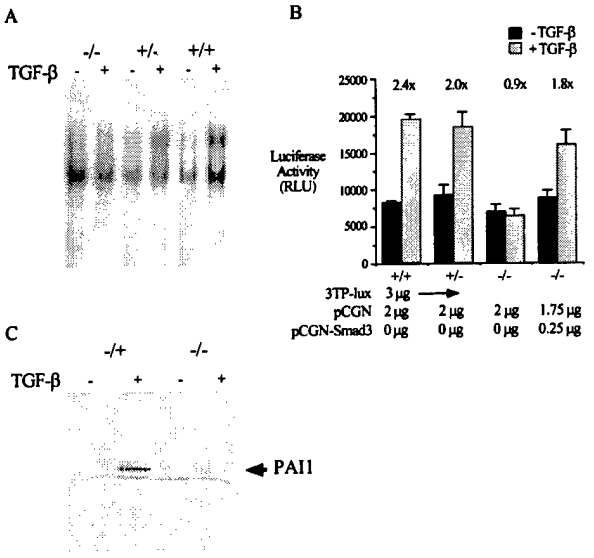

FIG. 4. Smad3 is required for TGF-β-mediated Smad-containing DNA binding complex formation and activation of 3TP-Lux in primary MEFs and for TGF-β-mediated induction of the PAI-1 gene in primary dermal fibroblasts. (A) Loss of a Smad-containing DNA binding complex in the Smad3 null MEFS. EMSAs were performed with nuclear extract from MEFs of the indicated genotype, either treated with TGF-β for 30 min or untreated, and a probe derived from the TGF-β-responsive region of the promoter-reporter construct, p3TP-lux. The arrow indicates the TGF-β-inducible DNA binding complex. (B) Smad3 is required for induction of the p3TP-lux reporter construct. The indicated DNAs were transfected into MEFs of the indicated genotype. Twelve hours after transfection, the cells were treated with 100 pM TGF-β for an additional 24 h, and TGF-β-induced luciferase activity (relative luciferase units [RLU]) from this reporter construct was assayed. Bars represent the average luciferase activity of duplicate transfections in a single experiment; error bars represent the standard deviation. Fold induction by TGF-β is indicated over each set of bars. (C) Smad3 is an integral component of the TGF-β-mediated induction of the endogenous PAI-1 gene. Smad3 heterozygote and null primary dermal fibroblasts were treated with TGF-β for 8 h. The arrow represents [^{35}S]methionine-labeled, extracellular matrix-associated PAI-1, assayed as described in Materials and Methods.

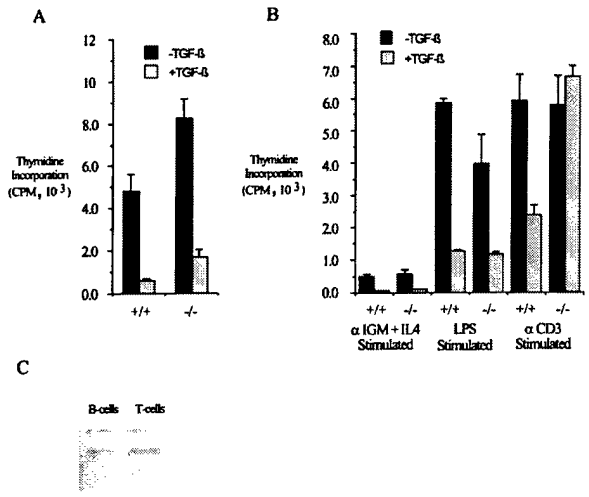

FIG. 5. Assay of TGF-β's effects in primary splenocytes reveals both Smad3-dependent and Smad3-independent growth-inhibitory signaling pathways. (A) Smad3 is not required for TGF-β-mediated growth inhibition in primary unstimulated splenocytes. Primary splenocytes were isolated from 8-week-old mice and cultured in the presence or absence of 100 pM TGF-β for 48 h. Cells were incubated with [^{3}H]thymidine for the last 4 h of culture, after which the splenocytes were harvested and ^{3}H incorporation was measured. Bars indicate the average of three identically treated wells for each growth condition; error bars represent the standard deviation. (B) Smad3 is required for TGF-β-mediated growth inhibition of αCD3-stimulated splenocytes. Primary splenocytes were isolated from 8-week-old mice and cultured in the presence of the indicated growth stimuli in the presence or absence of 100 pM TGF-β. Cellular proliferation was assayed by [^{3}H]thymidine incorporation as for panel A. (C) Smad3 is expressed in both B and T cells. Western blotting for Smad3 was performed on purified B and T cells from mature wild-type spleens.

growth-inhibitory effects of TGF-β in this system but also for the induction of this specific promoter reporter construct.

To assess the effect of Smad3 loss on the induction of an endogenous gene known to be transcriptionally regulated by TGF-β, we assayed the TGF-β-mediated induction of PAI-1 in primary dermal fibroblasts. As shown in Fig. 4C, the induction of PAI-1 by TGF-β seen in Smad3 heterozygote dermal fibroblasts is greatly reduced in the null cells.

Analysis of the immune cells derived from Smad3 null mice reveals a defect in TGF-β signaling. Having defined an essential role for Smad3 in TGF-β signaling in the fibroblast system, we next examined the cell types with highest Smad3 expression, those of lymphoid origin. We first examined the proliferation of splenocytes isolated from wild-type and null animals in the presence and absence of TGF-β. Interestingly, the proliferation of unstimulated primary splenocytes, consisting of a mixed B- and T-cell population, is inhibited by TGF-β regardless of mouse genotype when assayed by tritiated thymidine incorporation (Fig. 5A). Thus, in contrast to the MEF data presented above, Smad3 is not required for TGF-β-mediated inhibition of cellular proliferation in unstimulated splenocytes. In addition, the antiproliferative effects of TGF-β in primary splenocytes stimulated by LPS or αIgM plus IL-4, which specifically stimulate the proliferation of B lymphocytes through the activation of IgM receptor expressed only on the surface of B cells, is largely intact regardless of genotype. However, in primary splenocyte cultures stimulated with αCD3, an activator of the T-cell receptor complex, inhibition of proliferation by TGF-β is seen only in wild-type cultures (Fig. 5B). This difference in Smad3-dependent TGF-β responsiveness of the mixed splenocytes to a specific stimulus is not due to a difference in the expression pattern of Smad3, as demonstrated by Smad3 Western blot analysis of isolated T and B cells (Fig. 5C). Taken together, these data suggest that Smad3 plays a specific role in the inhibition of immune cell proliferation by TGF-β dependent on the nature of stimulus.

Little is known on the molecular mechanisms through which TGF-β inhibits proliferation of activated B and T cells, making it difficult to predict the role of Smad3 in this system. Concurrent with results obtained for a variety of cell types, TGF-β treatment of αCD3-stimulated wild-type spleen cultures leads to a decrease in Cdk2 kinase activity and a maintenance of Rb in a hypophosphorylated state. These effects occur with minimal change in the levels of Cdk2 and cyclin E and no change in the levels of p27. In contrast, TGF-β-mediated inhibition of Cdk2 kinase activity and maintained activation of Rb do not occur in the Smad3 null spleen cultures, further supporting the different growth properties of wild-type and null immune cells (Fig. 6A). Western analysis of various cell cycle components reveal no TGF-β-mediated change in the levels of the CDC25A phosphatase or the TGF-β-responsive Cdk inhibitors p15 and p21 (data not shown). Thus, the TGF-β growth-inhibitory pathway activated in αCD3-stimulated splenocytes represents a yet to be defined Smad3-dependent mechanism.

Subsequently, we examined the effects of TGF-β on cytokine production in αCD3-stimulated primary spleen cultures. In this system, TGF-β prevents the αCD3-mediated increase in the production of a number of different cytokines by the wild-type cells (Fig. 6B). This effect is even more dramatic than the growth-inhibitory effects of TGF-β on these cultures. As shown in Fig. 6B, the production of several cytokines, such as IL-2, IL-4, IL-5, IL-9, IL-13, and IL-15, is more than 80% reduced by TGF-β in wild-type splenocytes (Fig. 6C). In the Smad3 null culture, however, TGF-β clearly does not have the same effect on the levels of these cytokines as seen in the wild-type culture. This loss of TGF-β responsiveness is most marked in gamma interferon (IFN-γ), IL-2, IL-13, and IL-15 production. In addition, the non-TGF-β-treated levels of several cytokines are elevated in the null cultures. These results strongly suggest abnormal regulation of cytokine production in the absence of Smad3-mediated TGF-β signal transduction.

Since lymphocyte proliferation abnormalities are observed in vitro in Smad3 null cells, we next determined whether any abnormalities in the profiles of lymphocyte distribution could be observed in vivo by performing FACS analysis on Smad3 null and wild-type spleens and thymuses. As shown in Fig. 7, the thymuses of Smad3 null mice contain normal proportions of CD4 and CD8 single- and double-positive T cells, suggesting that thymic T-cell maturation is normal in Smad3 null mice. Similarly, the spleens from Smad3 null mice contain normal numbers and percentages of B cells and CD4 and CD8 single-positive T cells, suggesting that there is not an abnormal expansion of lymphocytes in the spleens of Smad3 null mice. In addition, we performed FACS analysis on bone marrow and peripheral lymph nodes for B- and T-cell populations and observed no difference between wild-type and Smad3 null mice (data not shown). Finally, we performed functional analysis of B and T cells by immunizing mice with various antigens and measuring both T-cell-dependent and T-cell-independent antibody production. Again, we did not observe any significant differences in antibody production between wild-type and Smad3 null mice (data not shown).

DISCUSSION

In an attempt to define the roles of Smad3 in TGF-β-mediated signal transduction, we have created mice harboring a

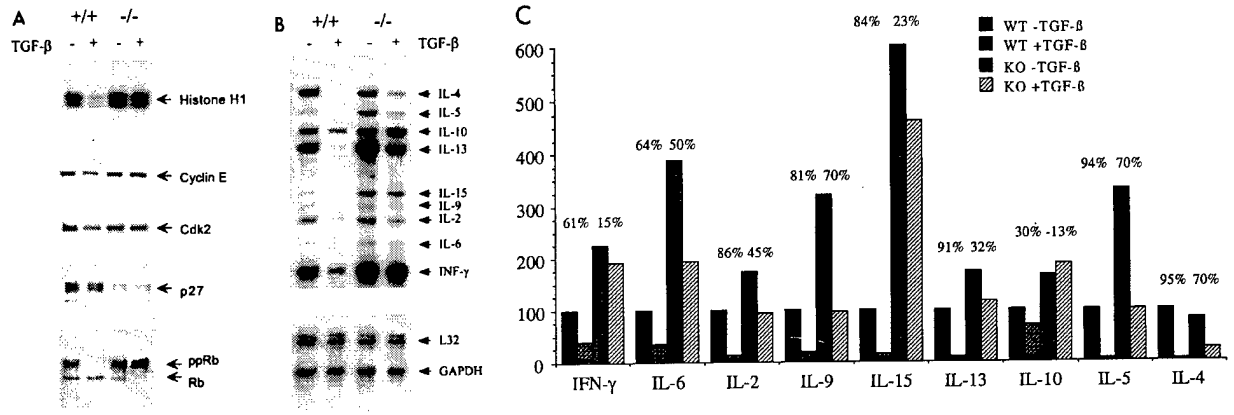

FIG. 6. TGF-β-mediated growth inhibition of αCD3-stimulated splenocytes is associated with a decrease in G_1 Cdk activity and cytokine expression. (A) Splenocytes were harvested from wild-type and knockout mice and cultured with αCD3 in the presence or absence of TGF-β. Cell lysates were prepared and subjected to Western blotting for cyclin E, Cdk2, p27, and Rb (lower panels). In addition, Cdk2 kinase activity was assayed by immunoprecipitation of Cdk2 and evaluation of its ability to phosphorylate the exogenous substrate, histone H1 (top panel). (B) Cytokine production was assayed on splenocytes from wild-type and Smad3 null mice treated as for panel A, using an RNase protection assay. The identity of each band is indicated on the right. L32 and *GAPDH* are controls for mRNA quantity and quality. (C) The intensity of the cytokine RPA bands in panel B was determined by densitometry. Plotted are the relative intensities of each band, with wild-type levels of each cytokine set at 100%.

targeted disruption of Smad3. The first striking finding is that Smad3 null mice are viable and survive to adulthood. The analysis of mice deficient in other Smad genes, however, has firmly established the role of this family of proteins in embryonic development. Mice with a targeted disruption of Smad4 display an early embryonic lethal phenotype at embryonic days 6.5 to 8.5. These embryos do not undergo gastrulation or express mesodermal markers, and they show abnormal visceral endoderm development (52, 61). Smad2-deficient mice also die early in development, at embryonic day 9.5, primarily due to a loss of anterior-posterior identity within the embryo. In the absence of anterior-posterior identity, the entire epiblast develops a extraembryonic mesodermal fate, failing to give rise to the three primary germ layers (55). In a separate study, Smad2 was found to play a role in mesoderm formation, left-right patterning, and craniofacial development (43). Additional support for the critical roles of the TGF-β superfamily of ligands and the Smad family of proteins in development has been established in studies of the *Xenopus* oocyte developmental system (17, 24).

In sharp contrast to mice harboring a targeted disruption of Smad2 and Smad4, the loss of Smad3 function, as we report here, has no discernible effect on embryonic development. It is conceivable that certain functions of Smad3 are redundant with, or compensated for by, that of Smad2. These two proteins are 90% identical at the amino acid level. Both proteins are inducibly phosphorylated by the TGF-β receptors, associate with Smad4, and undergo nuclear accumulation. One main difference is that Smad2 may be expressed as two alternatively spliced variants; one contains two inserts in the MH1 domain of the protein, rendering it unable to bind to DNA (51, 60), whereas the other, without the inserts, is structurally and functionally virtually identical with Smad3 (60). Thus, the molecular functions of Smad2 and Smad3 are most likely overlapping as well as distinct, since functional differences in the DNA binding properties and promoter activation by these molecules have been reported (28, 63, 65). Although we still do not know the expression patterns of the two variants of Smad2, clearly Smad3 cannot fully compensate for the severe defect in Smad2 null mice which may have lost the expression of both forms of Smad2. On the other hand, Smad3 may play a more exclusive role as an effector for TGF-β and possibly activin in adult tissues, whereas Smad2 with its two forms may function more globally in development and possibly in the adult as a signaling mediator of these two ligands.

A role for Smad3 in TGF-β-mediated growth inhibition. Our initial goal in these studies was to define the role of Smad3 in the regulation of cellular proliferation by TGF-β. Since previous studies on this topic have involved overexpression of Smads and the use of various tumor lines which likely harbor additional mutations, a role for the Smads in the regulation of proliferation remained uncertain. To this end, we have demonstrated that Smad3 is required for TGF-β-mediated growth inhibition in at least two cellular contexts: αCD3-stimulated primary splenocytes and primary MEFs.

The results from primary splenocyte cultures are particularly interesting in that TGF-β-mediated growth inhibition is dependent on Smad3 only under certain stimulated growth conditions. The proliferation of unstimulated, LPS-stimulated, and αIgM–IL-4-stimulated splenocytes is inhibited in response to TGF-β treatment in wild-type cells and to a nearly identical extent in Smad3 null cells. In contrast, a large reduction in TGF-β-mediated growth inhibition is seen in the Smad3 null splenocytes specifically when they are stimulated by αCD3. Thus, there appear to be both Smad3-dependent and Smad3-independent growth-inhibitory signaling pathways for TGF-β. We have also observed a similar defect in TGF-β-mediated growth inhibition in MEFs derived from Smad3 null mice. In these cells, the growth-inhibitory effect of TGF-β is largely absent, and this lack of TGF-β effect is most likely cell autonomous.

The molecular nature of the growth-inhibitory effects of TGF-β is one of its most studied properties. Through the work of a number of groups, a model has been put forward in which TGF-β regulates proliferation by inhibiting the activity of Cdk complexes. This function of TGF-β is likely due, in part, to its ability to increase the expression of the Cdk inhibitors p21 and p15, decrease the expression of a number of different cyclins, Cdks, the phosphatase CDC25A, and c-Myc, as well as regulate the activity of p27 (reviewed in reference 20). The signaling mechanisms of TGF-β-mediated growth inhibition vary significantly from one cell type to another. Unfortunately,

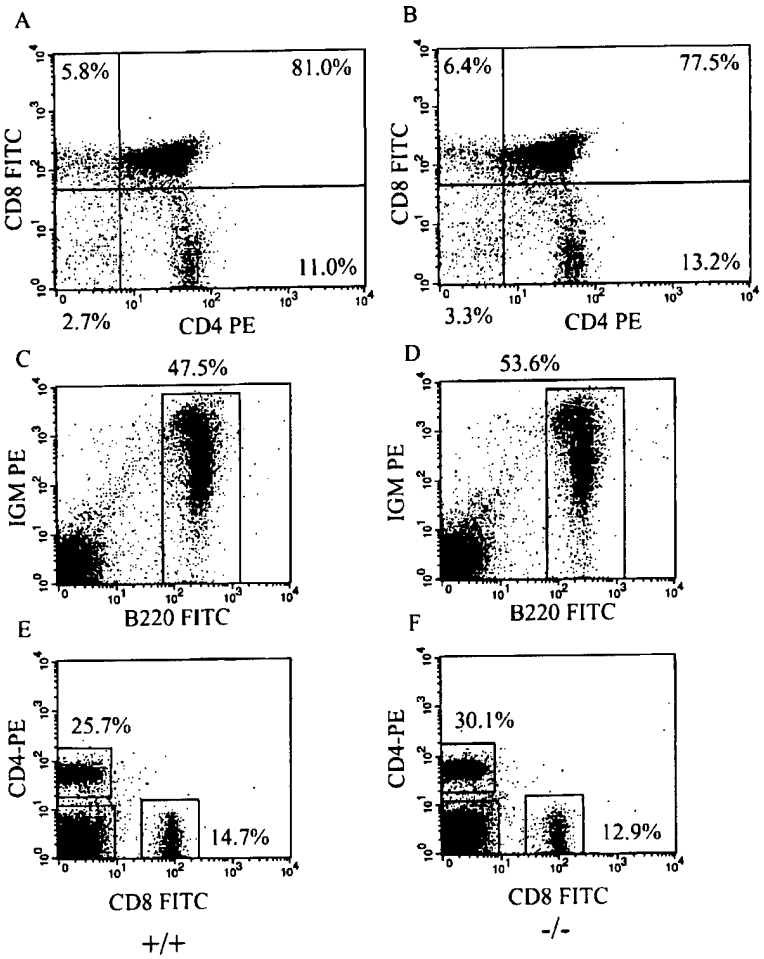

FIG. 7. FACs analyses of thymocytes and splenocytes isolated from wild-type and Smad3 null mice demonstrate normal T-cell and B-cell development. (A and B) Representative FACs analysis of wild-type and Smad3 null thymocytes, using αCD4-PE and αCD8-FITC. (C to F) Representative FACS analysis of wild-type and Smad3 null splenocytes, using the indicated conjugated antibodies. All data was gated for viable cells by the absence of 7AAD staining. Percentages represent the proportions of viable cells in each region or quadrant.

none of the previously described TGF-β-mediated growth-inhibitory pathways appear to be functioning in wild-type MEFs or αCD3-stimulated splenocytes. Specifically, MEFs and αCD3-stimulated splenocytes down regulate G_1 cyclin-Cdk complex activity without significant changes in the levels of p21, p15, p27, cyclin E, or Cdk2. Thus, Smad3 does not act through these defined downstream effectors to mediate the growth-inhibitory effects of TGF-β in these cells. Consequently, these findings suggest a novel Smad3-dependent growth-inhibitory pathway for TGF-β.

The work presented here is complemented by a recent report by Zhu et al., characterizing the phenotype of an independently created mouse line with a targeted insertion into the second exon of Smad3 (68). This group describes a high prevalence of colon tumor in the 129sv mouse background, and a lower prevalence of a less aggressive tumor phenotype in the 129-C57BL/6 hybrid mouse background. Although not experimentally addressed, it is an attractive hypothesis that this tumor formation occurs due to defects in TGF-β-mediated growth inhibition of the sort that we describe here. It remains to be determined, however, if these tumors arise from some other TGF-β–Smad3-dependent cellular effect or through a mechanism unrelated to TGF-β signaling. It is intriguing that we have not yet observed the 30% prevalence of colon tumors in our 129-C57BL/6 hybrid lines as in the reported study. This discrepancy may be due to differences in genetic background of the Smad3 null animals or even targeting strategies. It is also possible that a higher prevalence of tumors may still occur in our lines with longer time or when the mice with mixed genetic background are inbred into a pure 129 mouse line.

In addition to its antiproliferative role in the context of tumor suppression, TGF-β is a well-documented global inhibitor of immune system function. This function of TGF-β is evidenced by the phenotype of TGF-β1 null mice (9, 26). These mice present with a multifocal inflammatory disease, with lymphocyte infiltration into multiple organs and production of autoimmune antibodies (9, 13). The phenotype of these mice may be attributed to a loss of the antiproliferative effect of TGF-β1 on both B and T cells (22, 23). Given the fact that Smad3 is most highly expressed in the spleen and thymus, and the accumulating evidence that Smad3 is regulated by TGF-β, the development of an overactive inflammatory phenotype

similar to that of the TGF-β1 knockout mice may have been expected in the Smad3 null mice. This phenotype, however, is not observed.

These findings may be explained by the fact that under several conditions for assay of B- and T-cell cultures in vitro, the antiproliferative effect of TGF-β is intact in Smad3 null cells. Thus, under in vivo conditions, the proliferation of B and T cells may be appropriately inhibited under most circumstances by endogenous TGF-β. Since this is likely the case, a more subtle or incompletely penetrant inflammatory phenotype may still emerge in the Smad3 null mice. These findings also support a model in which although Smad3 is important in regulating the antiproliferative effects of TGF-β under certain conditions, TGF-β can also activate or use other Smad3-independent pathways to exert a growth-inhibitory effect.

A role for Smad3 in TGF-β-mediated gene responses. TGF-β can affect the expression of a number of different genes of diverse functions (46). The identification of Smads as sequence-specific DNA binding transcription factors supports the notion that the regulation of specific genes by TGF-β may be through the functions of Smad2, Smad3, and Smad4. Both 3TP-lux, a well-studied promoter reporter used for the analysis of TGF-β signaling, and the promoter of PAI-1, a highly TGF-β inducible extracellular matrix protein, contain Smad3-Smad4 DNA binding sites (11, 21, 63). Although the Smads have been implicated in the TGF-β-mediated induction of 3TP-lux and PAI-1, these studies are based largely on Smad overexpression and dominant negative studies, leaving the question of the physiological role for Smad3 in TGF-β-mediated gene activation unresolved (29, 35, 65). In addition, we have shown in a previous study that the Smad-DNA interaction is dispensable for the activation of 3TP-lux by TGF-β, bringing into question the role of Smad3 in the regulation of this promoter (63). Here we demonstrate that Smad3 is integral for transactivation of 3TP-lux and PAI-1, as their induction by TGF-β is reduced in the absence of Smad3. Interestingly, although Smad2 has been shown in the context of overexpression to activate 3TP-lux and PAI-1 (29), no compensation by Smad2 is observed in the Smad3 null fibroblasts.

In addition to the studies of 3TP-lux and PAI-1, we have investigated the role of Smad3 in the regulation of other genes by TGF-β. Specifically we provide evidence that the TGF-β-mediated down regulation of αCD3-stimulated cytokine production is Smad3 dependent. This suggests that Smad3 may play an important role in both the activation as well as the repression of gene expression. We have also evaluated the role of Smad3 in the regulation of several additional promoters by TGF-β in our Smad3 null model system. Specifically, we describe the role of Smad3 in the induction of c-Jun by TGF-β in a separate study (56). In this study we find that the c-Jun promoter contains a Smad3-Smad4 complex binding site and that Smad3 is required for the induction of c-Jun in MEFs. Thus, Smad3 may be specifically required for the activation of transcription from a subset of TGF-β-responsive promoters. In this regard, the Smad3 null MEF system provides a useful tool to define these genes which are regulated by TGF-β through Smad3. In doing so, we may be able to define novel genes or pathways which are at the root of the Smad3-dependent, TGF-β antiproliferative effects which we have described.

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The first two authors contributed equally to this work.

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